Analyst

CRITICAL REVIEW

Approaches towards Molecular Amplification for Sensing

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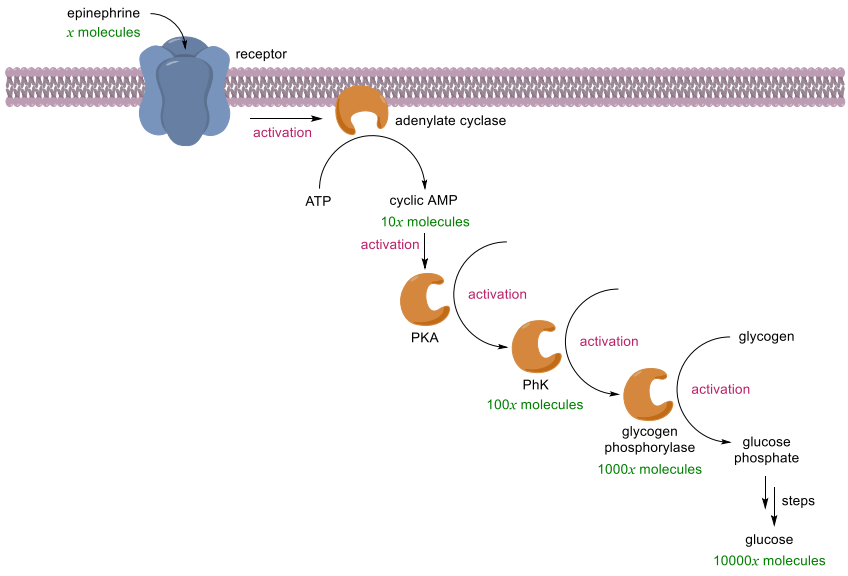
Diagnostic assays that rely on molecular interactions have come a long way; from initial reversible detection systems towards irreversible reaction indicator-based methods. More recently, the emergence of innovative molecular amplification methodologies has revolutionised sensing, allowing diagnostic assays to achieve ultra-low limits of detection. There have been a significant number of molecular amplification approaches developed over recent years to accommodate the wide variety of analytes that require sensitive detection. To celebrate this achievement, this comprehensive *critical review* has been compiled to give a broad overview of the many different approaches used to attain amplification in sensing with an aim to inspire the next generation of diagnostic assays looking to achieve the ultimate detection limit. This review has been created with the focus on **how** each conceptually unique molecular amplification methodology achieves amplification, not just its sensitivity, while highlighting any key processes. Excluded are any references that were not found to contain an obvious molecular amplifier or amplification component, or that did not use an appropriate signal readout that could be incorporated into a sensing application. Additionally, methodologies where amplification is achieved through advances in instrumentation are also excluded. Depending upon the type of approach employed, amplification strategies are divided into four categories: target, label, signal or receptor amplification. More recent, more complex protocols combine a number of approaches and are therefore categorised by which amplification component described within was considered as the biggest advancement. The advantages and disadvantages of each methodology are discussed along with any limits of detection, if stated in the original article. Any subsequent use of the methodology within sensing or any other application is also mentioned to draw attention to its practicality. The importance of amplification within sensing is wholly emphasised while perspectives on the future direction of the field are also shared.

Introduction

Amplification is the process whereby a small input signal is converted to a large output signal by means of an amplifier. Amplifiers are most commonly associated with electronics where they are used to increase the power of an electric signal for functions such as increasing audio volume. Amplifiers are also found within certain aspects of optics, fluidics and mechanics. In nature, living organisms use amplification for purposes such as visual excitation,[1] blood clotting,[2] and biosignalling.[3] Typically, these amplified physical responses to an external stimulus are achieved through enzyme cascade mechanisms (Scheme 1).[4] Specific enzyme cascades are initiated when a signalling molecule (*i.e.* a hormone) is detected by a signal receptor, which in turn activates an associated enzyme. Once activated, the enzyme can catalyse the production of multiple signal relay molecules, which can go on to activate more enzymes and so on, resulting in an enzyme cascade. An example of this can be found within the fight-or-flight response exhibited by animals where, in response to a perceived threat, epinephrine is



Scheme 1 Epinephrine-activated enzyme cascade for the amplified production of glucose.

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released by the body to activate an enzyme cascade for increasing blood sugar levels.[5] Through this powerful amplification mechanism, the detection of a single molecule of epinephrine by a receptor can lead to the rapid production of thousands of glucose molecules. Typically, amplifications over several orders of magnitude are observed within milliseconds.

The use of amplification is also found within many biological and chemical sensing techniques.[6] Since a single molecule is unable to generate a strong enough signal to be detected, some degree of amplification is paramount to achieving a detection protocol with high sensitivity. There is huge demand for the ability to detect of a large variety of different analytes in many areas of society but particularly within molecular diagnostics,[7] forensic analysis,[8] and environmental monitoring.[9] There is also increasing pressure to not only increase the sensitivity, but also improve the efficiency of currently existing analyte detection assays. As such, more and more amplification methodologies for a wide range of different analytes are constantly being developed. Analytes requiring detection are often structurally diverse in nature and the readout method chosen for detection also varies according to the application. To accommodate for this, many different amplification methodologies have been developed to for their specific detection with an appropriate sensitivity as well as providing a signal in the correct format for the readout method required. Despite the vast number of amplification methodologies published to date, they are typically achieved through one of the following four approaches; target amplification, label amplification, signal amplification or receptor amplification (Figure 1).

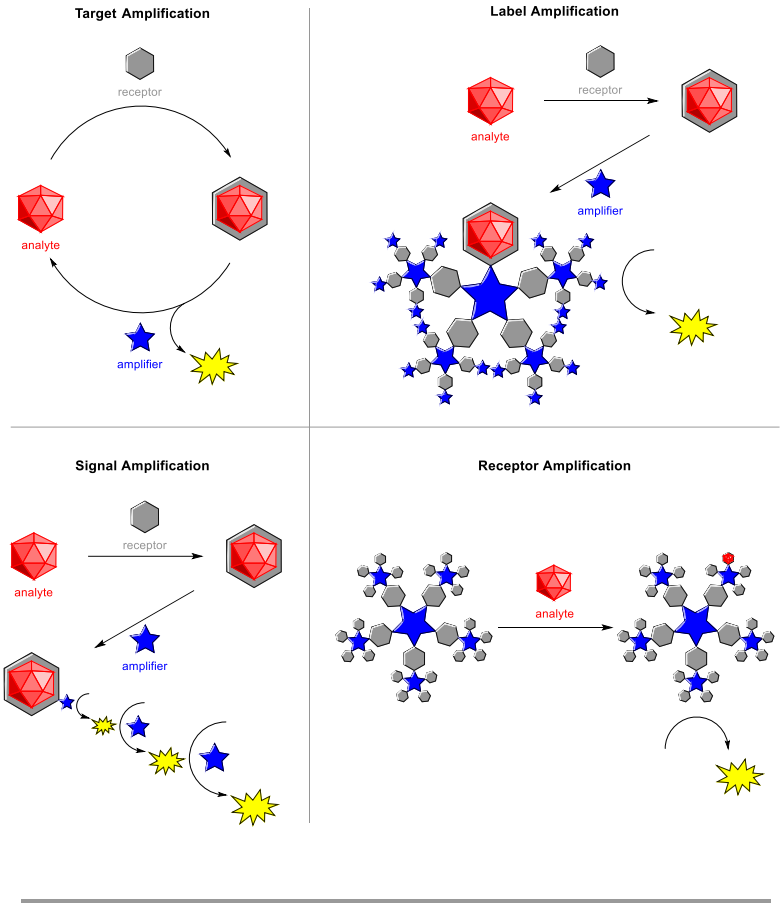


Figure 1 Four main approaches to achieving amplification within sensing.

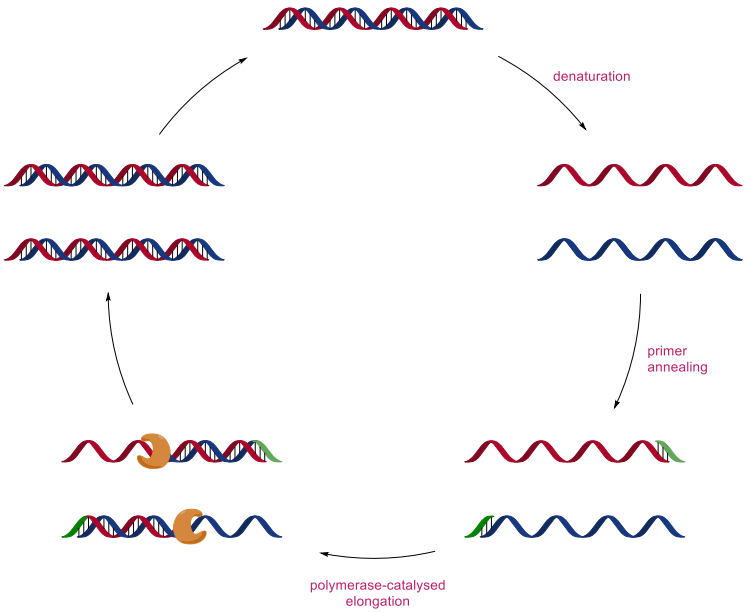
Target amplification involves increasing the number of analyte–receptor recognition events that occur *per* analyte. This can be done by increasing the analyte concentration prior to analyte recognition through analyte replication protocols, or by recycling the analyte after a signal has been produced post-analyte recognition. Additionally, analytes that can affect multiple labels or reporter molecules due to possessing catalytic properties themselves provide inherent target amplification within their corresponding detection assays. Label amplification focuses on increasing the number of labels *per* analyte recognition event. Since each label is responsible for providing a signal, the ability to amplify the number of labels that represent an analyte–receptor recognition event leads to a signal increase. This can be done using polymerisation or nucleic acid replication techniques or through the use of nanostructures, either directly increasing label numbers through multivalency or by providing a collective change in physical property to illicit a detectable response. Signal amplification is by far the most common approach to improving the sensitivity of a detection methodology and regards the use of amplifiers to enhance the signal produced from an analyte–receptor recognition event. Receptor amplification utilises a number of scaffolds to amplify the amount of receptors bound to the signal-generating mechanism. This increases the probability of achieving an analyte–receptor recognition event and, as a single binding event is able to affect the properties of the entire structure; an increased signal can be obtained in comparison to a single receptor system.

Although the majority of amplification-related publications usually fall within one of the above categories, there are a few that are not exclusively bound to such parameters and, for simplification, are categorised by which best defines the approach described by the authors. This becomes increasingly apparent in more recent manuscripts where the desire to achieve even greater assay sensitivity has been accomplished through a combination of different approaches.

Target Amplification

Target Replication

One strategy to improve the limit of detection (LOD) of an assay is to simply increase the number of target molecules present. If only few target molecules are present then an efficient replication technology, specific only to the analyte, would increase the



Scheme 2 Target replication: the polymerase chain reaction (PCR) cycle.

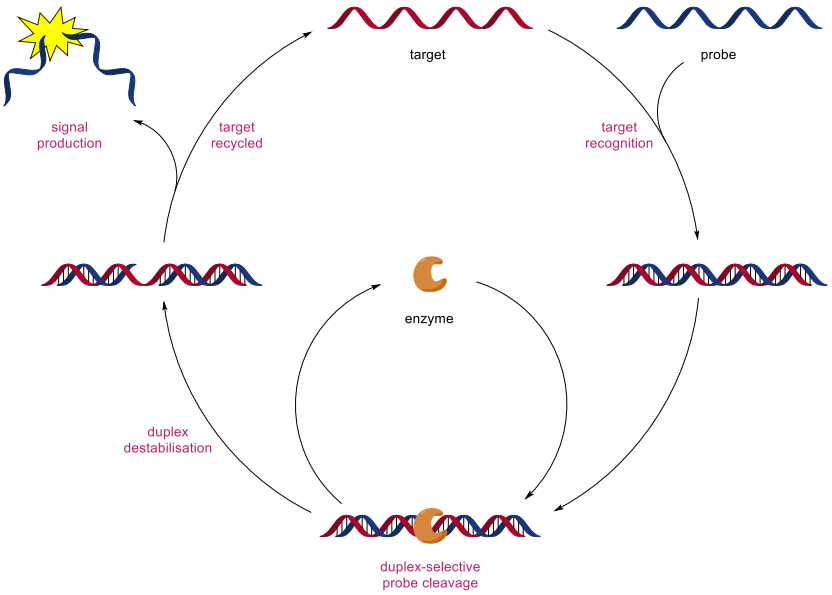
concentration of target molecules to a detectable level. Traditionally, target replication has been specifically developed for the amplification of nucleic acids and there have been a substantial number of different strategies developed for this purpose.[10] One of the most widely-known sensing amplification methodology ever produced is a prime example of target replication and is known as the polymerase chain reaction (PCR).[11]

PCR is used to amplify target strands of deoxyribonucleic acid (DNA) prior to its detection and is carried out over a number of cycles (Scheme 2).[12] Each cycle begins with denaturation of the double-stranded DNA at elevated temperatures in order to obtain the target DNA in its single-stranded form. From here, DNA primers are then annealed indicating the start of the specific sequence of DNA due to be replicated. Starting from the primer, an enzyme known as DNA polymerase elongates the nucleic acid sequence base-by-base, complementary to that of the single-stranded DNA. After replication has finished, the cycle is complete and two identical copies of double-stranded DNA are yielded. These steps can then be repeated until amplification yields a detectable concentration of target DNA, typically between 15 to 40 cycles. As with every PCR cycle the amount of target DNA present is doubled, then DNA amplification through PCR is exponential; one of only a handful of sensing methodologies that truly exhibits exponential amplification. Because of this powerful amplification, and the fact that the process can be automated using thermal cyclers, PCR has been incorporated into many areas of science where sensitive DNA detection is paramount, such as forensics, evolutionary molecular biology and molecular diagnostics.[13] Current research into PCR has been focused towards avoiding the use of thermostable polymerases and thermal cyclers in order to reduce the time, and improve the efficiency of the protocol. A number of isothermal nucleic acid amplification technologies have since emerged,[14–15] which allows for nucleic acid sensing to be performed in the point-of-care (POC) setting.[16] One limitation of PCR however is that it is specific only to the amplification of nucleic acids. Target amplification methodologies that demonstrate the exponential amplification of other analytes as yet remain undiscovered.

Target Recycling

In the majority of cases, analytes to be detected are unable to be replicated as a means of providing amplification. Target amplification can still be achieved however, if the analyte can be recycled after a signal has been produced through an analyte recognition event. By doing this, one target molecule is able to act upon multiple probe molecules that provide the detectable signal. This can be described as target recycling and is another useful method of providing target amplification.

One such example of this is enzyme-assisted target recycling (EATR) and similarly to PCR, is also used primarily for the detection of nucleic acids (Scheme 3).[17] In this approach, single-stranded target DNA hybridises with probe oligonucleotides, which are usually in a vast excess compared with the target. The resulting double-stranded DNA is acted upon by an enzyme that selectively cleaves the probe and in the process, releases a detectable signal.[18]

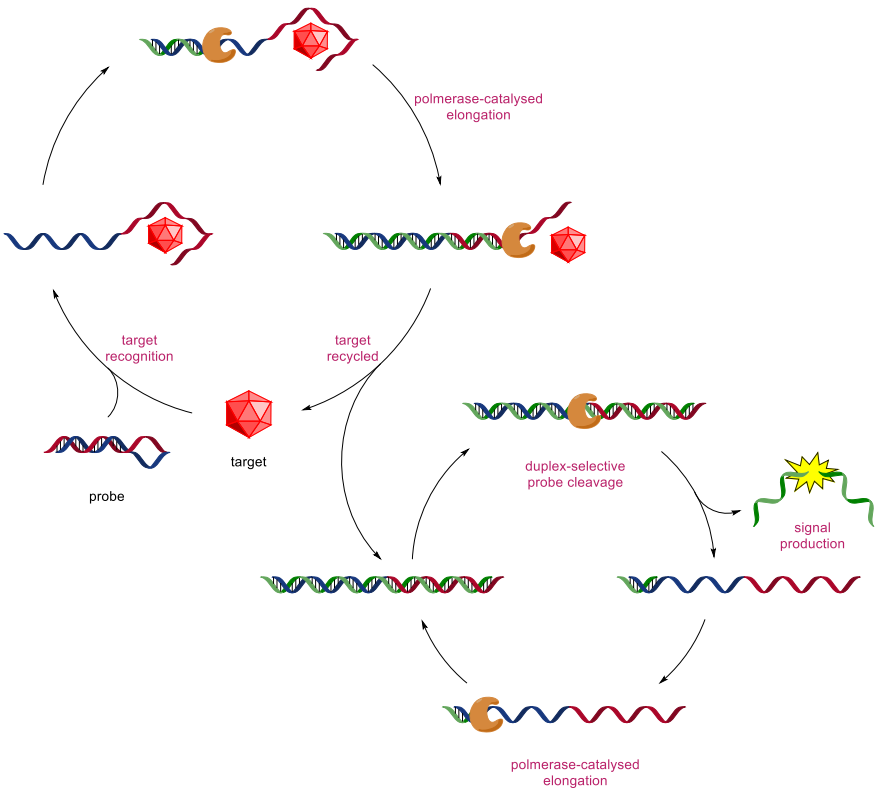


Scheme 3 Enzyme-assisted target recycling of nucleic acids.

Enzyme-cleavage also results in complex destabilisation, which then undergoes separation releasing the single-stranded DNA target capable of reacting further with more probes oligonucleotides. Although EATR produces a linear amplification compared with the exponential amplification provided by PCR, fewer false-positives occur since the procedure is less sensitive towards DNA contaminants. Additional benefits of EATR over PCR include an improved robustness and reduced cost, making it a useful amplification methodology for affordable POC diagnostics. The same concept can also be achieved with ribozymes (nucleic acid enzymes) instead of enzymes, allowing for autocatalytic variants of this methodology to become possible.[19]

Subsequent improvements to EATR amplification methods have been through the development of self-cleaving probes, thus removing the need for enzymes entirely.[20] In their non-hybridised form, the probes are stable towards any inter- or intramolecular reactions with itself, giving no signal in the absence of the target. In the presence of the target, target-assisted self-cleavage (TASC) occurs, destabilising the complex and leading to recycling of the target along with the production of the signal, typically *via* fluorescence. This 'reagent-free' approach towards DNA sensing has inspired the development of DNA-templated reactions where target DNA can act as a catalyst for turning substrates into products.[21] Coupling substrate turnover with signal production allows for the reaction to be monitored over time and DNA templates have been shown to provide >200-fold increase in signal within 30 seconds over a non-templated reaction, and can achieve a turnover number >1500 with a LOD of 0.5 pM.[22]

This methodology is not restricted to just the detection of nucleic acids as Huang *et al.* have recently shown that target recycling can be applied to the detection of antibiotics (Scheme 4).[23] This amplification procedure actually utilises two recycling pathways that operate in sequence to minimise background signal production yet deliver ultrasensitive detection. For selective detection of the target antibiotic, ampicillin, the group use a hairpin DNA structure containing an aptamer probe. In the presence of the target, the hairpin is opened allowing the binding of a DNA primer and subsequent complementary strand replication to occur through the use of a polymerase enzyme. This process both recycles the target, allowing it to react with further probes and produces a double helix that then enters its own EATR cycle producing multiple copies of short single-stranded DNA. This amplified sequence is then able to bind to electrochemically-labelled complementary sequences, preventing them from being detected at the electrode. Thus, a positive sample is observed in this case by a signal decrease. The use of a DNA aptamer for analyte recognition delivered excellent selectivity for the target, even in the presence of similar structures such as penicillin, and the LOD for this system was calculated to be 1.09 pM after a 4.5 hour amplification period which boasts a 100-fold improvement over existing methods.



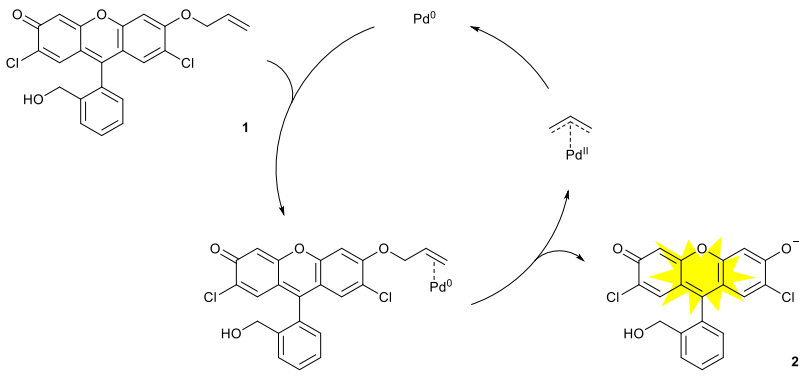
Scheme 4 Quadratic enzyme-assisted target recycling for small molecule detection.

These versatile target recycling methodologies have also found use within applications other than sensing. For example, two EATR cycles can cross-trigger one another to achieve simultaneous self-amplification for use towards a DNA-based artificial biochemical circuit.[24] Additionally, cascaded catalytic nucleic acids have been used to control reactivity, perform logic operations and assemble complex structures.[25]

Target Catalysis

Another target amplification strategy is to utilise any catalytic attributes that the analyte being detected may possess. Therefore, target catalysis as an amplification methodology is typically used for the detection of trace metal contaminants found in pharmaceutical ingredients and waste water streams. Due to the significant increase in the number of metal-catalysed cross-coupling reactions used in the pharmaceutical industry over recent years, metal impurities within drug compounds are becoming commonplace and are considered to pose a potential health risk.[26] Palladium is most commonly used within these processes and

consequently, restrictions have been placed upon the amount of residual palladium allowed to be found in medicinal compounds.[27] Unsurprisingly therefore, the development of sensitive detection methodologies for palladium has been widely sought-after.



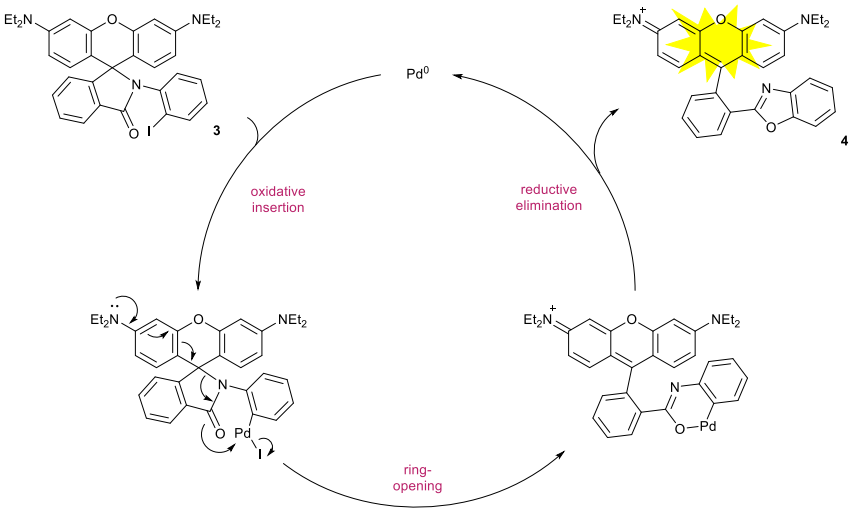
Scheme 5 Fluorogenic detection of palladium using a catalytic deprotection strategy.

Koide *et al*. were the first to exploit palladium's catalytic ability as a method of providing amplification for its detection.[28] Non-fluorescent compound **1** was designed with an allyl moiety for analyte recognition which, only in the presence of Pd^0, undergoes deallylation *via* a Tsuji–Trost-like mechanism (Scheme 5).[29–30] Quantitative detection of the released, highly-fluorescent product **2** can be performed using a standard fluorometer allowing for a LOD of less than 1 ppm of palladium *per* mg sample. This efficient example of target amplification was successfully shown to detect residual Pd^0 in pharmaceutical products as well as impregnated within used glass reactor vessels. In addition, the probe has been used within a high-throughput method for the detection of palladium within ore samples towards the more efficient and economical mining of precious metals.[31]

As Pd^{II} is readily reduced to Pd^0, both oxidation states of palladium are detected by this method. However, by changing the mechanism used for palladium detection to a metal-catalysed aromatic Claisen rearrangement,[32] selective detection of Pd^{II} over Pd^0 could be achieved.[33] The similar reactivity of palladium and platinum however can cause selectivity issues, but through careful selection of the solvents used in the assay and by maintaining a specific pH, this can also be overcome.[34] Improvements to this detection methodology include the introduction of reducing agents and phosphine ligands in order to increase the catalytic activity of the palladium, which in turn leads to increased sensitivity of the assay.[35–36] Furthermore, a bis-allyl ether derivative of **1** has been very recently reported that can significantly reduce background fluorescence and as such, delivers a >300-fold signal increase within a 5 minute reaction period.[37]

Alternative moieties have also been shown to be selective for palladium detection as a propargyl derivative of compound **1** has been shown to be able to monitor palladium accumulation in living systems *via* fluorescence.[38] Probes with different fluorescent scaffolds have also been used for palladium detection within cells.[39,40] Among these, naphthalimide-derived probes are considered the most accurate since fluorescence detection is ratiometric.[41] Palladium sensing is not confined to just allyl or propargyl deprotection as Jun and Ahn have utilised the catalytic cross-coupling capability of palladium to develop a 'switch-on' fluorescent detection method (Scheme 6).[42] In this example of target catalysis, non-fluorescent probe **3** was designed comprising of an aryl iodide for analyte recognition and a spirolactam derivative of a rhodamine dye, a common pro-fluorescent scaffold.[43] Oxidative insertion into the carbon–halide bond induces ring-opening of the spirolactam, and subsequent reductive elimination provides highly-conjugated fluorescent product **4** whilst simultaneously regenerating the target. This highly sensitive technique allows for the specific detection of palladium at concentrations as low as 2 ppb. Following the success of these initial reports, there has been extensive research into the development of alternative probes for both the colourimetric and fluorescent detection of palladium and platinum based upon this catalytic amplification concept.[44–45]

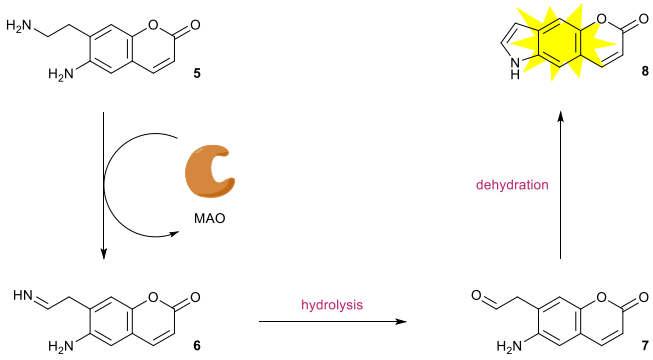
The use of target catalysis for the detection of other metals that also demonstrate catalytic activity have similarly been attempted. For example, both the Koide,[46–47] and Ahn,[48] groups have employed their switch-on fluorescence sensing methodologies for the detection of mercury. Since Hg^{2+} selectively activates alkynes and vinyl ethers towards hydrolytic cleavage, protecting their respectively-developed



Scheme 6 Fluorogenic detection of palladium using a catalytic cross-coupling strategy.

fluorophores with these functional groups would give access to non-fluorescent mercury substrates. Although this is indeed the case, the mechanism for the mercury-promoted ether cleavage is not catalytic and involves consumption of the metal ion, usually released as an organomercury compound. As the target does not exhibit catalytic attributes, target catalysis is not observed in these cases and thus, fall outside the scope of this review. Other sensing methodologies which appear to employ target catalysis, such as the fluorescent detection of Co^{2+},[49] Cu^{+},[50] and Cu^{2+},[51] actually require super-stoichiometric amounts of the analyte to deliver full signal switch-on.

After the definition of "click" chemistry was proposed,[52] the copper-catalysed alkyne–azide cycloaddition (CuAAC) reaction,[53–54] a catalytic derivative of the cycloaddition originally developed by Huisgen *et al.*,[55] has become the synonymous example and has been employed within numerous applications over recent years.[56] Manipulation of non-fluorescent reagents to afford a highly fluorescent product under the reaction conditions has led to the development of the fluorogenic click reaction.[57] Although not specifically designed for sensing, this methodology does utilise target catalysis to generate an amplified fluorescent product in the presence of catalytic amounts of copper. Due to the efficiency of CuAAC, Q. Wang *et al.* could screen a large range of coumarin,[58] and anthracene,[59]-derived profluorophores in a combinatorial fashion to quickly identify which coupling partners combine to give products with the highest quantum yields. As the functional groups are inert and stable under biological conditions, as well as the high yields and excellent regioselectivity of the reaction, this reaction has understandably been designed for biolabelling purposes and protocol has contributed significantly towards the development of reaction-based small-molecule fluorescent probes used for chemoselective bioimaging.[60]

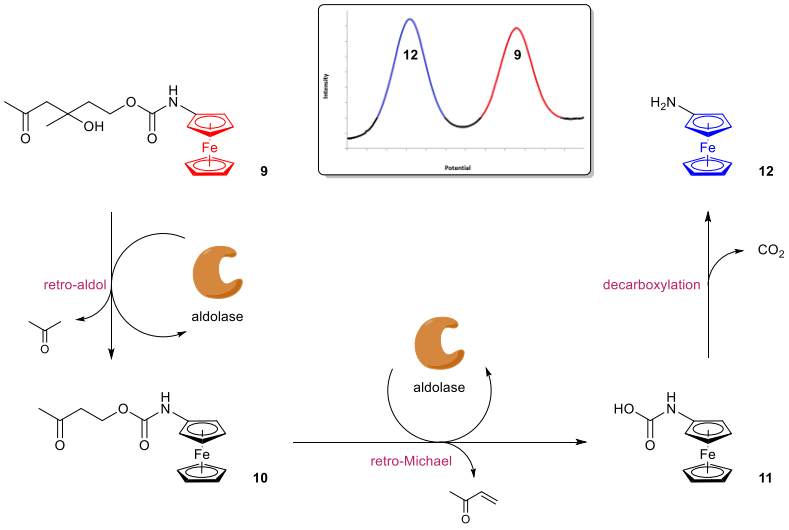


Scheme 7 Fluorogenic enzyme assay for the detection of MAO.

In addition to the sensitive detection of metals, the detection of enzymes are equally important especially within high-throughput screening for drug discovery as well as enzyme engineering.[61] Since a single enzyme is capable of generating multiple reporter molecules, amplification in the form of target catalysis is inherent within enzyme detection assays.[62] The simplest and most effective strategy is to couple enzyme-responsive functional groups with water-soluble chromogenic or fluorogenic moieties to obtain enzyme substrates that selectively exhibit a change in physical property in the presence of an enzyme.[63] For example, Sames *et al.* designed a range of fluorogenic probes for oxidoreductase enzyme detection based on this concept.[64] During their investigation, they found that one probe in particular was a highly selective substrate for 3-α-hydroxysteroid dehydrogenase (HSD), an enzyme responsible for the activation and deactivation of steroid hormones.[65] This enabled fluorescence imaging to be performed on human cells in order to monitor the up-regulation of HSD in response to panaxytriol,[66] an active compound found in red ginseng; a common ingredient within herbal medicines.[67]

By changing the enzyme-responsive trigger unit, the group were able to simply achieve a highly selective 'switch-on' fluorescent assay for monoamine oxidase (MAO) (Scheme 7),[68] an enzyme associated with neurological and psychiatric diseases.[69] In this enzyme detection assay, compound **5** was exposed to MAO which catalyses the aerobic oxidation of amines to the corresponding imines.[70] Imine **6** is then hydrolysed under the aqueous conditions required for enzyme activity to give aldehyde **7**, which then undergoes intramolecular cyclisation and dehydration to afford highly fluorescent indole **8**. Although not a particularly sensitive system due to the slow turnover rate of the enzyme, it did allow for the direct and continuous measurement of MAO activity in mitochondria, utilising target catalysis for amplification.

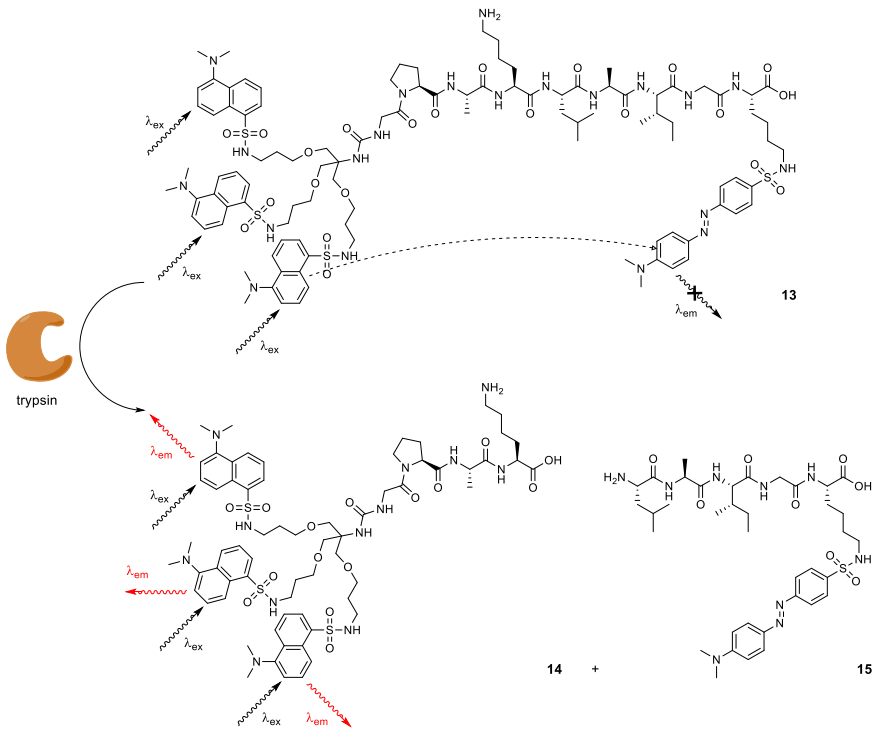
Although comprising the significant majority, enzyme detection methods are not solely achieved using colour or fluorescent signal production.



Scheme 8 Electrochemical enzyme assay for the detection of aldolase activity.

Electrochemistry has been gaining significant popularity due to increased specificity, speed, portability and low cost in comparison with the aforementioned optical detection techniques.[71] In addition, samples do not need to be purified or transparent prior to detection, the instruments does not require complex calibration to maintain accuracy and miniaturisation has allowed electrochemical detection to be easily incorporated into point-of-care (POC) biosensors.[72]

An example of target catalysis used for amplification within an electrochemical detection assay can be provided by Shabat *et al.* for monitoring aldolase activity (Scheme 8).[73] In this development, ferrocene-labelled substrate **9** was synthesised and exposed to a catalytic antibody that expresses aldolase activity. In its presence, substrate **9** first undergoes a retro-aldol reaction to afford compound **10**, followed by a retro-Michael elimination to give carbamic acid **11**. Under the assay conditions, this unstable acid spontaneously decarboxylates to achieve aminoferrocene **12**. Importantly, substrate **9** and product **12** were found to have significantly different oxidation potentials and as such, could be distinguished electrochemically. Despite requiring two enzyme-catalysed reactions to afford the detectable product, current changes could still be measured at an antibody concentration as low as 0.33 μM. This elegant procedure can also be extended to the ratiometric electrochemical detection of other enzymes such as alkaline phosphatase (ALP) through simple modification of the enzyme-responsive functional group upon the substrate.[74]



Scheme 9 FRET enzyme assay for the detection of trypsin.

Label Amplification

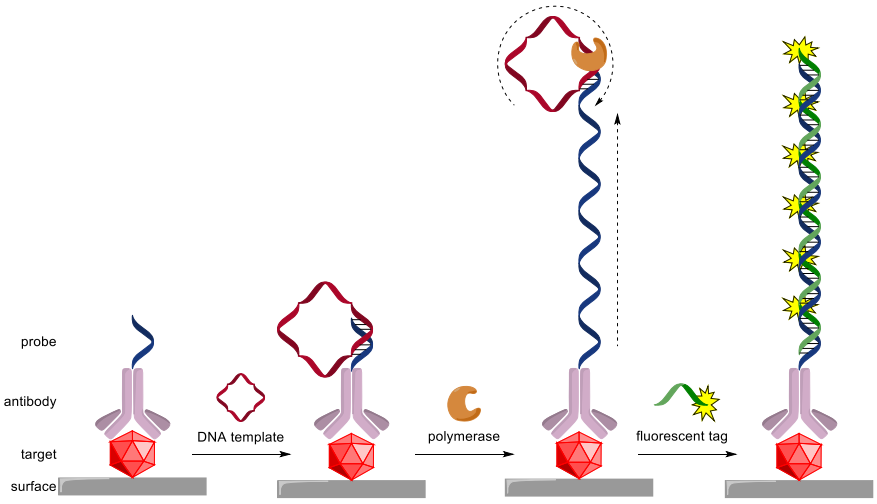
Since the majority of analytes requiring detection do not possess catalytic attributes or can be easily replicated, target amplification is therefore a difficult amplification strategy to successfully implement. An alternative approach is to amplify the number of signal-producing labels that correspond to an analyte–probe recognition event. The concept is simple; increasing the number of signal-producing labels *per* analyte recognition event would lead to an amplified signal output compared with a single-labelled probe. The concept was successfully demonstrated exemplified by Ternon and Bradley who developed an amplified assay for fluorescent protease detection through the use of multiple valent fluorophores (Scheme 9).[75]

In this approach, Förster resonance energy transfer (FRET) was utilised through the construction of peptide substrate **13** containing a tri-fluorophore label at one end and a fluorescence quencher at the other.[76–77] Thus, in the absence of the enzyme target, intramolecular fluorescence quenching occurs and a fluorescent signal is therefore not observed. However, in the presence of the target protease, trypsin, cleavage of the peptide occurs which fragments the substrate preventing intramolecular quenching from occurring and consequently, a fluorescent signal is observed. In comparison to a single fluorophore-labelled substrate, compound **13**, which contains three fluorophores, was capable increasing the signal-to-noise ratio of the assay by a factor of 4.6. Although amplification was observed through this method, the assay is limited by the number of fluorophores bound to the substrate, in this case just three. As such, label amplification methodologies developed since have looked at alternative ways at increasing the number of labels *per* analyte–probe recognition event and typically, these methods are found to be more effective when employed after such an event.

Label Replication

One approach to providing label amplification is to apply a replication methodology subsequent to an analyte–probe recognition event where the probe is labelled with an easily-replicated moiety. Similarly to target amplification approaches, nucleic acid replication methodologies are ideal and a derivative of PCR known as rolling circle amplification (RCA) is commonly used.[78] RCA is an isothermal nucleic acid amplification protocol which has found use in many applications within chemical biology, materials science and medicine.[79] Typically, circular single-stranded DNA, called a template, is linearly replicated hundreds or thousands of

times to give an extremely long strand of single-stranded DNA. Since the same DNA sequence is repeated, multiple complementary probe strands are able to hybridise to the amplified single-stranded DNA label enabling sensitive detection normally through fluorescence.[80]

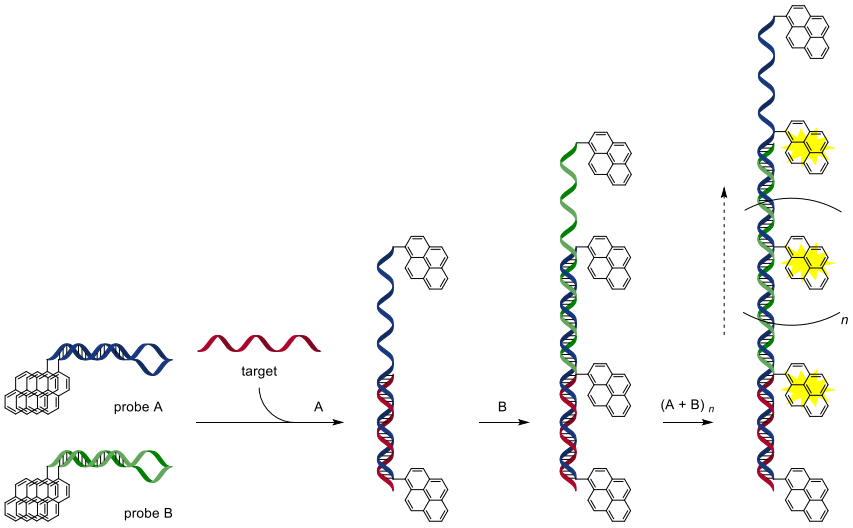


Scheme 10 Label amplification using RCA in a DNA-labelled immunoassay.

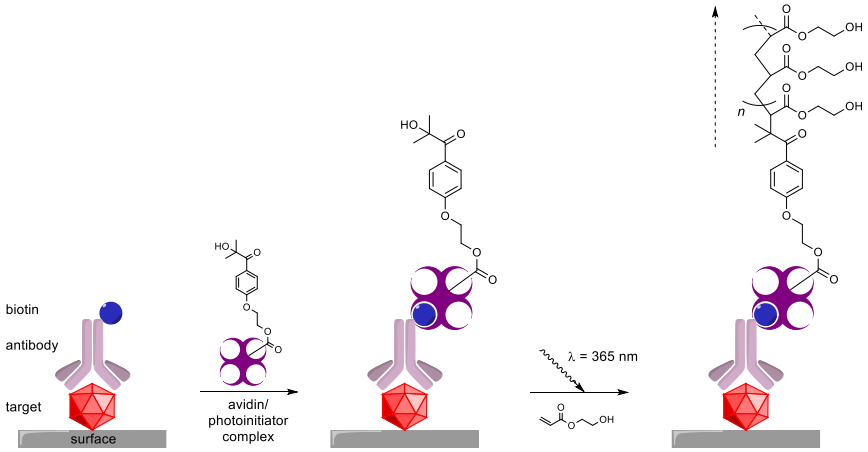
The application of RCA to immunoassays, known as immuno-RCA, allows for the sensitive detection of proteins through the labelling of probe antibodies with a specific DNA sequence (Scheme 10).[81] In the presence of the analyte, hybridisation with the circular template enables replication of the label DNA sequence using RCA, which can then be detected by fluorescence through the hybridisation of multiple complementary DNA sequences tagged with a fluorophore. This strategy allows for an enormous ratio of fluorescent molecules *per* label, and therefore *per* analyte, and as such, delivers a highly sensitive detection system. Jiang *et al.*, for example, achieved a LOD of 0.9 fM for the detection of human immunoglobulin G (IgG) using immuno-RCA.[82]

RCA has also been combined with oligonucleotide-functionalised nanoparticles for sensitive cancer cell detection by electrochemical methods with an obtained LOD of 10 cells *per* mL.[83] Increasing the sensitivity of DNA-labelled immunoassays can also be achieved by using PCR as the amplification technique.[84] Termed immuno-PCR, this technique delivers 100 to 10000-fold increase in immunoassay sensitivity and has been applied to the sensitive detection of tumour markers, viral proteins and bacterial pathogens.[85–86] However, to allow the label to be amplified by PCR, cleavage of the DNA label from the analyte–probe complex is required which can be difficult to perform efficiently without denaturising the complex and creating unwanted contaminants that can hinder the PCR amplification process.

A reagentless label replication strategy known as the hybridisation chain reaction (HCR) was developed by Dirks and Pierce, that involved two stable DNA hairpin structures that can coexist in solution until selectively triggered by the analyte.[87] Once triggered, the two strands of DNA react with one another in an alternating end-over-end fashion. By introducing 2-aminopurine (2AP), a fluorescent adenine analogue,[88] into the structure of one of the hairpin structures, HCR can be measured through fluorescent quenching. Alternatively, labelling both ends of both hairpin structures with pyrene enables a switch-on fluorescent DNA detection derivative (Scheme 11).[89] Not only is this amplification method isothermal and enzyme-free, it is also both highly selective, as base-pair mismatches, base deletion or base insertion are all discriminated from the target, and highly sensitive as DNA detection is in the femtomolar range. These highly advantageous attributes has enabled HCR to be used for *in situ* amplification and fluorescent imaging within vertebrate embryos.[90] The versatility of such an approach has also been demonstrated as by replacing the pyrene labels with nanoparticles, the detection of nucleic acids,[91] as well as enzymes,[92] can be achieved using electrochemical methods.



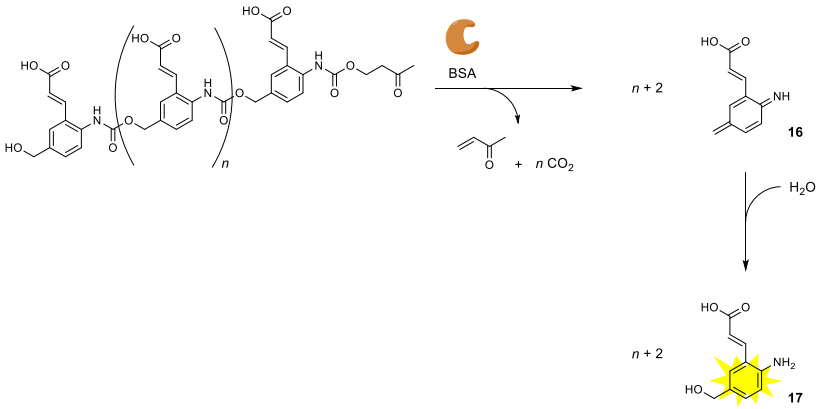
Scheme 11 HCR approach to label amplification.



Scheme 12 Polymerisation-based label amplification.

A synthetic approach to label replication was developed by Bowman *et al.* who achieved an amplified response through molecular recognition-selective polymerisation (Scheme 12).[93] In this approach, surface-bound biotin-labelled analytes are tagged with a macrophotoinitiator *via* an avidin complex. Irradiation of the surface in the presence of a monomer-containing solution induces polymerisation selectively from the photoinitiators, which therefore only occurs in the presence of the analyte. A 10 minute exposure to 365 nm wavelength light was enough to initiate polymers with a thickness greater than 100 nm, which is significant enough to see with the unaided eye and allowed a DNA concentration of 0.5 fmol to be easily detected. It has been estimated that an amplification factor of between 10^6 to 10^7 can be achieved through this methodology.[94] This polymerisation-based amplification has also been applied to the detection of the influenza virus through macrophotoinitiator-labelled immunoassays.[95] To provide quantitative measurements, as opposed to the quantitative visual readout, fluorescent nanoparticles can also be incorporated into the polymer enabling DNA detection,[96] and antibody detection,[97] to be achieved through fluorescent polymerisation-based amplification.

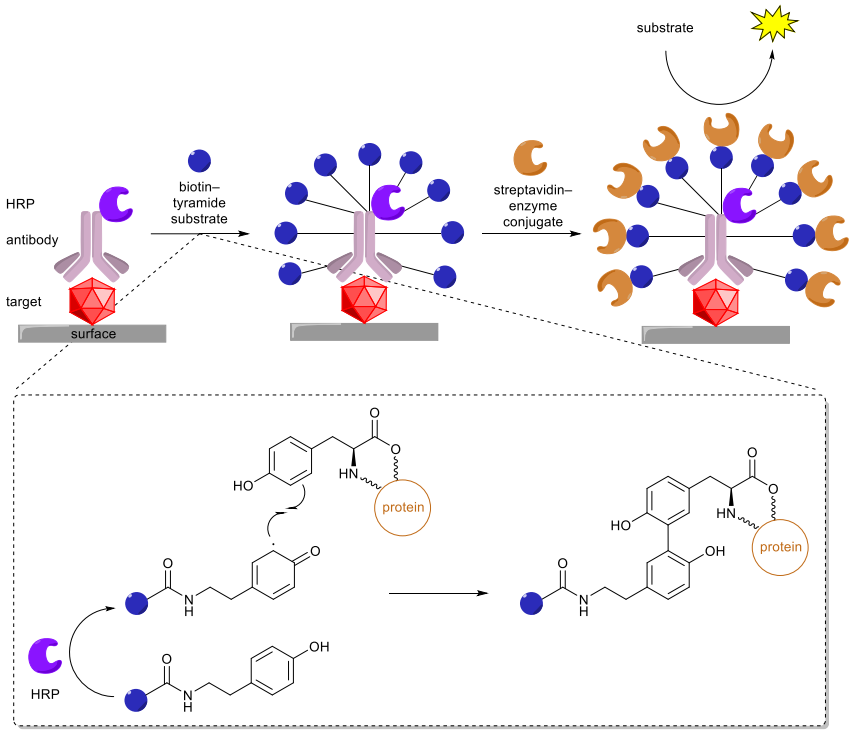
In a conceptually opposite approach, self-immolative polymers that undergo triggered depolymerisation in the presence of a specific analyte have been developed by Shabat *et al.* for catalytic protein detection (Scheme 13).[98] Typically, polymer chains comprised of between 15–20 pro-fluorescent monomer units and an analyte-responsive trigger unit at the head were synthesised. Cleavage of the head group occurs selectively in the presence of bovine serum albumin (BSA),[99] triggering continuous head-to-tail depolymerisation,[100] with subsequent release of the monomer units and trapping of these aza-quinone-methide intermediates **16** with water, generates aniline **17** with a consequential turn-on of fluorescence. Although full consumption and full elimination of the polymers is slow, a 1 mgmL^{-1} concentration of BSA could easily be distinguished from the background after an hour. Different monomers can easily be incorporated into the polymer allowing multiple signals to be released simultaneously upon a single trigger cleavage.[101] This has led to self-immolative polymers being used for a number of biomedical applications as well as for amplification within diagnostic assays.[102–103]



Scheme 13 Integer label amplification through triggered head-to-tail depolymerisation.

Label Multiplication

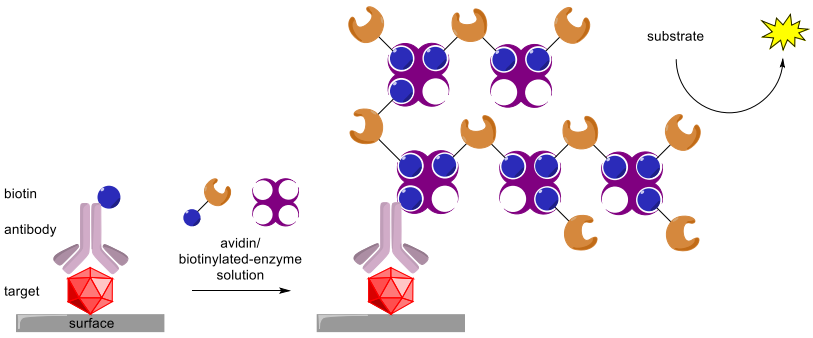
In the majority of instances, label replication is typically restricted to the use of nucleic acids as labels as it relies upon nucleic acid amplification procedures such as PCR and RCA. However, these methods are dependent upon the successful conjugation of the DNA label to the antibody, which can be difficult to achieve without denaturising the antibody. Also, polymerisation methods can be irreproducible and quantification of analyte concentrations can be difficult. A more general approach to providing label amplification can be provided though multiplying the number of labels present after an analyte–receptor recognition event. In this method, the label that indicates the presence of the target is able to capture multiple identical probes in the adjacent vicinity, thus multiplying the number of labels *per* target. This is exemplified by an amplification procedure designed to increase immunosensitivity known either as tyramide signal amplification (TSA),[104] or catalysed reporter deposition (CARD).[105]



Scheme 14 Catalysed reporter deposition (CARD).

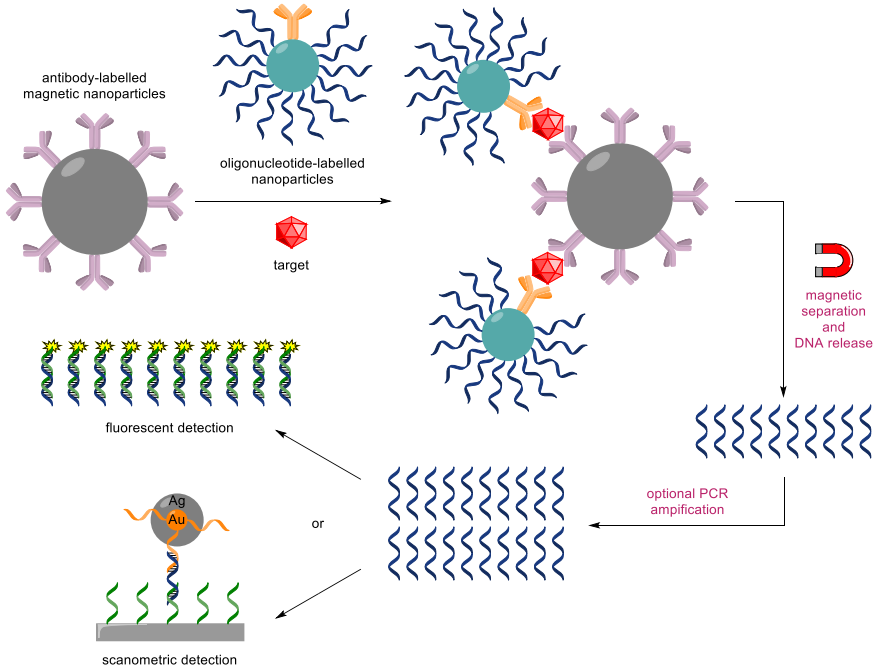
Typically, CARD is used to multiply the number of enzyme labels that represent the presence of a target protein after an initial analyte–receptor complex has been labelled with an enzyme (Scheme 14).[106] In this example, the target analyte is bound to a solid surface and identified with the corresponding antibody labelled with the enzyme horseradish peroxidase (HRP). In the presence of a biotin-labelled tyramine-derived substrate, the enzyme catalyses the formation of highly reactive tyramine radicals that react readily with any tyrosine units found within the adjacent protein structures.[107] This results in the macromolecular structure being covered with many biotin molecules, each of which is able to bind another enzyme *via* its streptavidin conjugate utilising the high binding affinity between biotin and streptavidin.[108] By employing this approach, one target is able to bind multiple enzyme labels which, upon addition of a pro-fluorescent substrate, leads to an amplified fluorescent output. This method is reported to deliver improved sensitivity in comparison to other probe multiplication methods towards increasing immunoassay sensitivity such as the avidin–biotin complex (ABC) approach.[109]

The ABC approach to label multiplication typically utilises the multivalent characteristics of the streptavidin tetramer avidin, in order to increase the number of labels that represent a target molecule (Scheme 15).[110] Similarly to CARD, target proteins are identified by appropriate antibodies after to being adsorbed onto a solid surface. Instead of being labelled with an enzyme however, the antibodies are labelled with biotin. The addition of a solution consisting of avidin and biotin-labelled HRP leads to a vast interlinked biotin–avidin network containing multiple peroxidase enzymes *per* complex. As a result, when the enzyme network is exposed to a solution containing the enzyme substrate, an amplified response is observed compared to an immunoassay that uses just one enzyme *per* target analyte. This has led to the ABC approach to label amplification to be used for a number of applications such as affinity chromatography and enzyme localisation studies, as well as for diagnostics.[111] However, like many immunoassay amplification methodologies, this system is susceptible to high background rates due to non-specific adsorption. Not only this, but the procedure also adds multiple manipulation steps in comparison to standard immunoassays.[112]



Scheme 15 Avidin–biotin complex (ABC) approach to label amplification.

The concept of synthesising structures possessing multivalent characteristics in order to multiply the number of labels corresponding to a single target molecule has inspired the development of many other label amplification approaches. In particular, nanostructures have found considerable success as label amplifiers, especially within biosensors, due to their small size yet large collective surface area.[113–115] For example, Rusling *et al.* described the use of carbon nanotubes as the label amplifier within an electrochemical immunosensor for sensitive cancer biomarker detection.[116] In this approach, the carbon nanotubes function as a bridge between the antibody used for target identification, and the enzyme labels. The amount of enzyme labels *per* binding event was calculated to be approximately ninety, which allowed for a LOD of 4 pgmL^{-1} for the cancer biomarker, prostate specific antigen (PSA). Similarly, this technique was also used for the attomolar detection of DNA, which equates to just 80 copies.[117] Gold nanoparticles, which are easier to functionalise than carbon nanotubes, have also been used as the label amplifier.



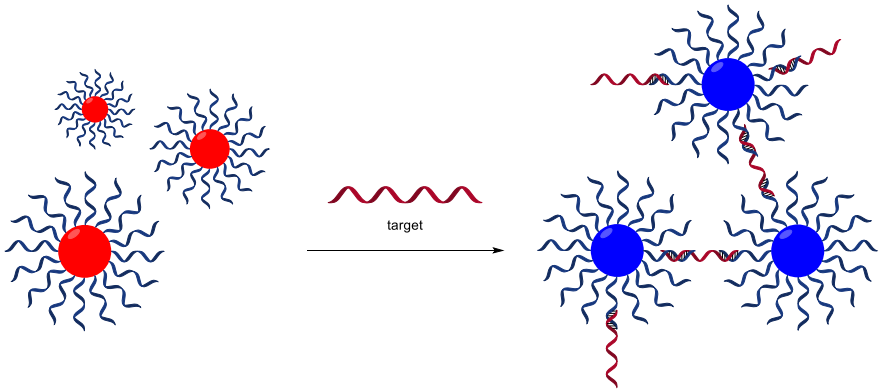
Scheme 16 Detection of a target protein using nanoparticle-based bio-barcode probes.

The enzyme label to binding event ratio was found to dramatically increase through this change, since the use of 1 µm diameter gold nanoparticles allowed up to five hundred thousand enzyme labels to be attached *per* nanoparticle. Due to this massive label amplification, a LOD of 1 fgmL^{-1} was obtained for the detection of cancer biomarker interleukin-8 (IL-8).[118]

The ability to attach a large number of labels upon a single nanoparticle and that gold nanoparticles in particular are easier to functionalise through strong covalent gold–thiol bonds, has seen them incorporated into a large number of chemical and biological sensing methodologies for label amplification.[119] One extremely sensitive approach was developed by Mirkin *et al.* who functionalised gold nanoparticles with multiple copies of oligonucleotides with identical sequences to obtain what are known as "bio-barcodes".[120] These bio-barcode nanoparticles were then used as label amplifiers for the sensitive protein detection (Scheme 16).[121] In this approach, antibody-functionalised magnetic capture nanoparticles are used to identify target proteins which, in the presence of the analyte, are labelled with the bio-barcodes through the formation of a highly-stable sandwich complex. The complex is then separated from the mixture magnetically and the oligonucleotide labels are released from the nanoparticle either thermally by hydrolysis or chemically, using dithiothreitol (DTT).[122] If necessary, the relea sed oligonucleotides can also be amplified by PCR to further increase the sensitivity of the assay. Detection is often achieved scanometrically on a solid surface,[123] or by fluorescence in solution using fluorophore-labelled probes.[124] By functionalising the magnetic capture nanoparticle with DNA instead of antibodies, the bio-barcode sensing approach can be applied towards DNA detection. The sensitivity of this approach allows for target DNA detection with a LOD of 0.5 aM, which approximately equates to just 10 copies of DNA *per* 30 µL sample.[125] The bio-barcode amplification approach also exploited the versatility of nanoparticles through the simultaneous use of magnetic capture nanoparticles with different probes and different label nanoparticles which allows for the multiplex detection of either DNA,[126] or proteins.[127]

Label Aggregation

Another approach to label amplification can be demonstrated by an analyte's ability to affect a large number of labels in a collective fashion, as opposed to amplifying the label post an analyte–probe recognition event as seen in replication or multiplication strategies. Rather than having one reporter molecule being made responsible for the production of the signal with amplification efforts being made to increase the number of these molecules *per* analyte, it is the strategy of label aggregation to harness all the reporter molecules available to create the signal and by changing the physical properties of a collective signal in the presence of the analyte, significant amplification can be achieved. This type of collective response to minor changes in conditions is analogous to colourimetric pH indicators where a slight increase or decrease in acidity is enough to shift the equilibrium of the system, which can result in a dramatic colour change. In a similar manner, the optical properties of gold

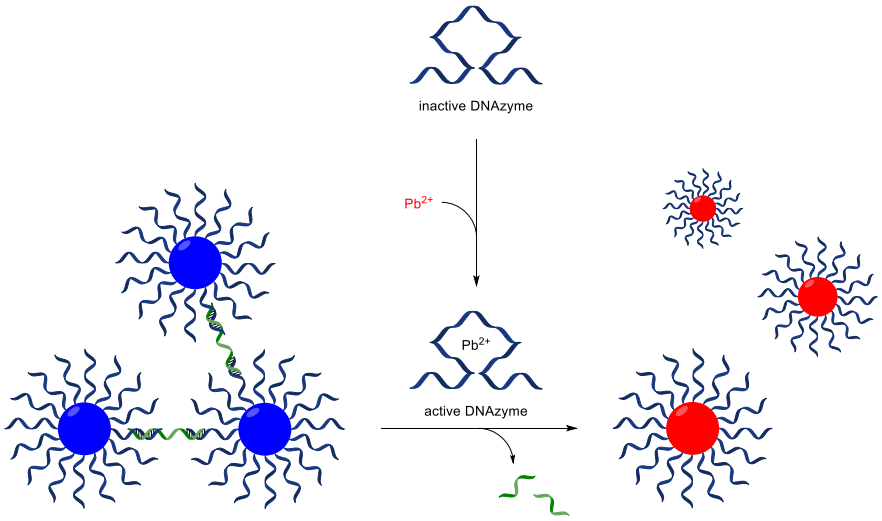


Scheme 17 Gold nanoparticle label aggregation for colourimetric DNA detection.

nanoparticles are known to be dependent upon the distance between each nanoparticle, which is attributed to the surface plasmon resonance (SPR) of gold.[128] Manipulation of this physical property phenomenon for label aggregation within a sensing methodology was first provided by Mirkin *et al.* for the colourimetric oligonucleotide detection (Scheme 17).[129]

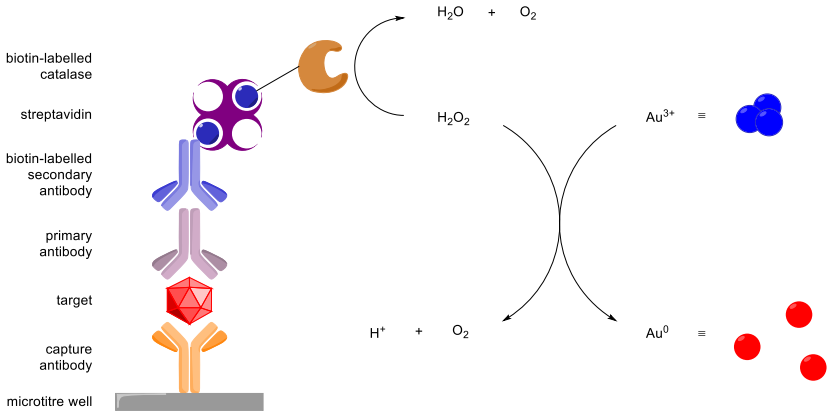
In this approach, ≈13 nm gold nanoparticles were constructed and derivatised with mercaptoalkylated probe oligonucleotides to afford a nanoparticle-containing solution with a characteristic red colour since the distance between particles is significantly greater than the particles themselves. In the presence of target oligonucleotides, hybridisation at both ends of the target causes significant aggregation of the nanoparticles and the subsequent decrease in interparticle distance results in a colour change to blue. This simple DNA detection and label amplification method was found to be surprisingly selective as a single base-pair mismatch could be distinguished from the complementary target. Sensitivity was also high allowing for a LOD of ≈10 fM of oligonucleotide to be easily detected.

In a similar but opposite approach, Liu and Lu utilised the disaggregation of gold nanoparticles towards colourimetric lead ion detection (Scheme 18).[130] In this setup, an oligonucleotide probe was used to aggregate gold nanoparticles together through hybridisation at both ends. In the presence of lead, inert DNAzymes are activated which results in cleavage of the oligonucleotide substrate serving as the linkage between the nanoparticles. The dissociation of the nanoparticles results in an easily-visualised colour change from blue to red. Since DNAzymes can be synthesised to be very selective for particular cofactors,[131] this method was shown to be highly selective for lead in the presence of a host of other metal ions. The label aggregation provided by this methodology provided excellent sensitivity with a LOD of 100 nM lead concentration shown to be detectable by the unaided eye.



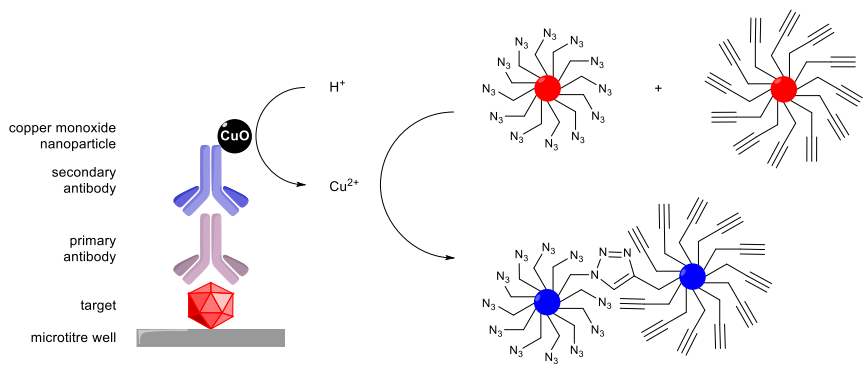
Scheme 18 Gold nanoparticle label deaggregation for colourimetric lead detection.

Gold nanoparticulate label amplification has also been applied to enhancing the sensitivity of enzyme-linked immunosorbent assays (ELISAs). ELISAs are typically protein detection assays that were initially developed as a safer and inexpensive alternative to immunoradiometric assays (IRMA) that used radioactive labels.[132] Indirec t or 'sandwich' ELISA is the most common format of choice for medical diagnostics as although requiring extra steps in the protocol compared with other formats, it is the most selective since the procedure utilises two antibodies; one absorbed onto the solid-phase for antigen capture and another conjugated to an enzyme for antigen labelling.[133] To increase ELISA sensitivity, de la Rica and Stevens developed a plasmonic ELISA for the visual detection of disease biomarkers (Scheme 19).[134]



Scheme 19 Plasmonic ELISA employing gold nanoparticulate label amplification for the colourimetric detection of disease biomarkers.

In this example, a traditional sandwich ELISA labelled with catalase, an enzyme that catalyses the breakdown of hydrogen peroxide, was constructed. In the presence of the target, and therefore the enzyme, hydrogen peroxide is broken down to water and molecular oxygen which allows the solution containing Au^{3+} ions to form ill-defined nanoparticles that aggregate together leading to a blue colour to be developed. In the absence of the target, hydrogen peroxide remains in solution and reduces the Au^{3+} ions in the solution to Au^0. This leads to the formation of regular, spherical nanoparticles that do not aggregate together and cause a red colour to be developed. This example of gold nanoparticle label amplification in conjunction with enzyme signal amplification



Scheme 20 Copper oxide nanoparticle-labelled immunoassay employing gold nanoparticulate label amplification for colourimetric HIV antigen detection.

allowed for a LOD of 1 agmL^{-1} for the detection of PSA and enabled the visual detection of HIV antigens at concentrations that were undetectable by a traditional nucleic acid-based test. This has since given rise to the development of a number of biodiagnostic tests that utilise plasmonic nanomaterials for both amplification and signal production.[135] However, successful and accurate signal production is highly dependent on a freshly prepared and precise concentration of hydrogen peroxide.[136]

Another example of gold nanoparticulate label amplification being applied to protein detection has been described by Jiang *et al.*, who used copper oxide nanoparticle labels to catalyse the aggregation of gold nanoparticles for the detection of human immunodeficiency virus (HIV) antigens (Scheme 20).[137] Here, a direct immunoassay was constructed where the antigen was adsorbed onto the surface of the microtitre well and identified first with a primary antibody, then with a secondary antibody tagged with copper oxide nanoparticles. In the presence of the nanoparticles, and therefore the analyte, the introduction of a strong, acidic solution to the assay dissolves the copper nanoparticles, releasing Cu^{2+} ions into the system. Once released, the Cu^{2+} ions can catalyse a click reaction between two sets of gold nanoparticles; one set covered with terminal alkynes and the other covered with alkyl azides.[138] After a significant number of gold nanoparticles have been knitted together, the aggregation causes the colour of the solution to change from red to blue. In the absence of the antigen, the copper catalyst is not produced and the gold nanoparticles remain separate keeping the detection solution red. Although not explicitly stated, the LOD obtained for this detection method was said to rival that of a traditional fluorescence ELISA.

Label multiplication methods such as CARD and the ABC approach both suffer from a lack of versatility as there are only a limited number of labels available for amplification. Nanostructures compensate for this as their versatile nature has made them popular label amplification tools within biosensing. However, they are often cumbersome to prepare and suffer from high backgrounds due to oversensitivity from non-specific adsorption. The use of controlled aggregation for label amplification is a simple yet effective strategy for amplification within sensing and typically has the benefit of a colour change which can be easily visualised. Despite having examples exhibiting powerful amplification, label amplification techniques are often analyte-specific and are restricted to the specific type of diagnostic assay they are designed for in order to achieve successful amplification.

Signal Amplification

Signal amplification is the process whereby the signal generated from an analyte–probe recognition event is amplified by an external amplifier. In this context, an amplifier is considered as a component which can produce or turnover multiple reporter molecules *per* amplifier and are therefore typically synthetic catalysts or enzymes. Since no such amplification component under this description is apparent within many simple turn-on fluorescence or colourimetric assays, these sensing methodologies predominantly used within molecular logic gate systems,[139–142] indicator displacement assays (IDAs),[143–145] and reaction-based indicator systems,[146] are therefore excluded from this review. These particular classes of assay have become subject of a number of specific, recent reviews and readers interested in these areas are directed towards the respective references.

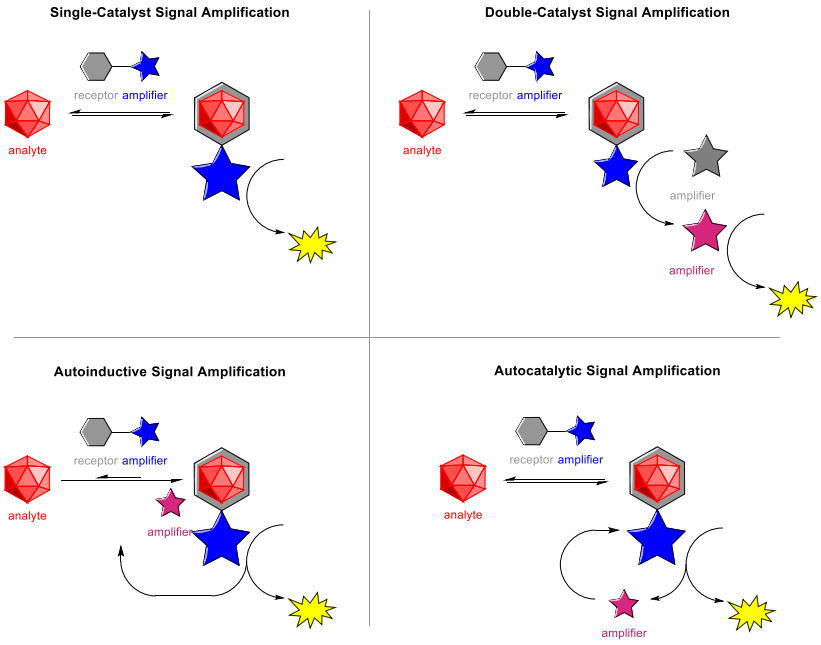


Figure 2 Four main approaches towards molecular signal amplification.

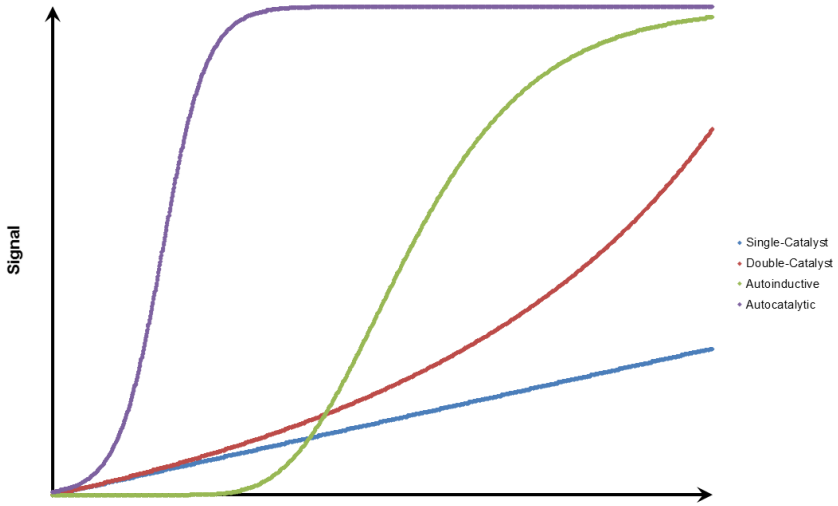


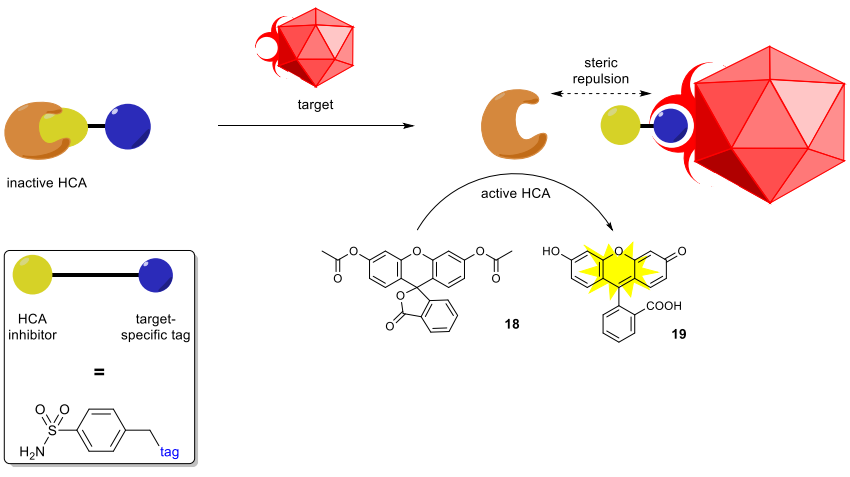
Figure 3 Typical signal amplification profiles observed for the different signal amplification mechanisms.

Signal amplification is the most popular amplification method employed in sensing as it is often the easiest amplification methodology to design and implement, as amplification does not involve amplifying the target or the label. Due to the high number of examples, signal amplification approaches can be divided into four distinct areas based on the mechanism of amplification: single-catalyst, double-catalyst, autoinductive and autocatalytic (Figure 2). As one would expect, the reaction profiles displayed by these signal amplification mechanisms are typically markedly different (Figure 3). However, it is not a case of which one provides the biggest or fastest signal as, dependent upon the application, one type of signal amplification may prove more suitable than another.

Initial developments towards implementing signal amplification within diagnostic assays were focused upon using a single amplifier such as an enzyme or a catalyst, which provides a linear increase in signal over time. A natural succession from this led to the successful combination of two consecutive signal amplifier s to achieve dual-enzyme, dual-catalyst or even hybrid enzyme–catalyst amplification cascades, which often display quadratic-type amplification profiles. The constant strive to attain even greater assay sensitivity has led to the development of complex auto-amplification strategies, such as autoinductive and autocatalytic signal amplification, where the signal-amplified products themselves can increase the rate of signal production. Autoinductive signal amplification is a system whereby the products can indirectly accelerate the rate of a kinetically meaningful step of the signal amplification mechanism whereas autocatalytic signal amplification is the process whereby the products themselves directly serve as catalysts for their own production.[147] As a result, both auto-amplification types exhibit sigmoidal amplification profiles with autocatalysis demonstrating exponential signal amplification. Again, choosing the appropriate signal amplification protocol for a diagnostic assay is dependent upon a number of factors including, but not limited to, the type of analyte being detected, the medium the amplification is being performed in, the length of time after analyte recognition that a signal is required and the readout method used. For example, an assay requiring the lowest LOD possible would look to elect an autocatalytic signal amplification method whereas an assay requiring greater accuracy would look to employ a single-catalyst signal amplification strategy.

Single-Catalyst Signal Amplification

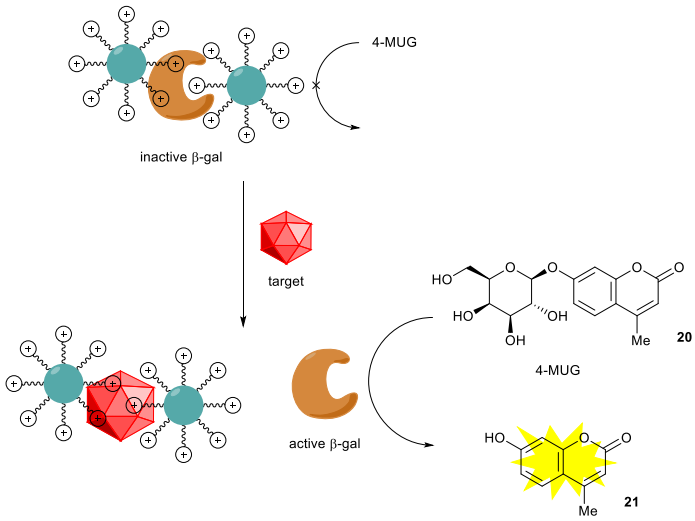
Signal amplification methodologies typically use enzymes or synthetic catalysts as amplifiers due to their ability to generate multiple product molecules without themselves being chemically altered. By designing a detection system whereby the enzyme or catalyst indicate the presence of the target and product formation results in a change in physical property, then catalyst or enzyme activity, and therefore the target, can be easily detected.[148] As such, early signal amplification methodologies focused on the selective activation of the amplifier in the presence of the target analyte. Typically, this is achieved either through intrasteric or allosteric activation. Intrasteric activation involves direct competition between the analyte and an inhibitor for the catalyst or enzyme active site and allosteric activation



Scheme 21 Intrasteric enzyme activation through steric repulsion for signal-amplified protein detection.

involves structural alteration of amplifier conformation which results only from recognition of the analyte at a site on the amplifier, other than the active site.[149] Examples of both allosteric and intrasteric activation of enzymes, as well as allosteric and intrasteric activation of synthetic catalysts have all been successfully demonstrated for the detection of a wide variety of analytes.

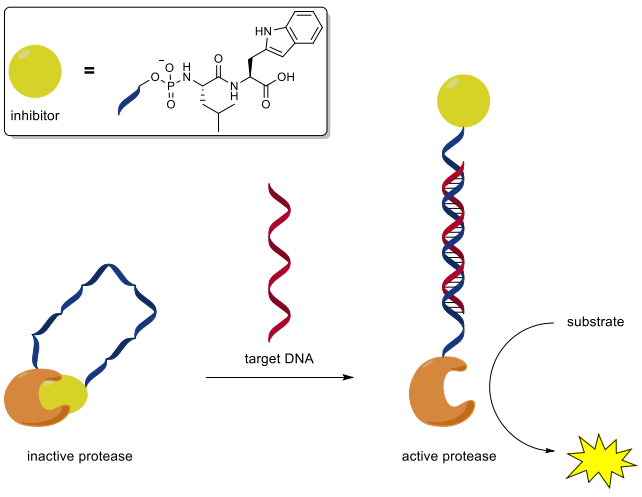
The very high turnover rate of enzymes under physiological conditions have made them popular signal amplifiers within sensing methodologies and varying enzyme activation methods have been employed to enable selective analyte detection. Intrasteric activation of enzymes is one particular method of activation and works through initial blocking of the active site of the enzyme with an inhibitor which restricts enzyme activity. Removal of the inhibitor by an analyte-recognition event restores enzyme activity which can be detected by the addition of an appropriate enzyme substrate. A very recent development by Tan *et al.* provides a perfect example where the activity of human carbonic anhydrase (HCA) is intrasterically regulated through a strongly bound benzenesulfonamide inhibitor which is labelled with a protein-specific tag (Scheme 21).[150] In the presence of the target protein, a binding event with the tag occurs which plucks the inhibitor out of the HCA active site in order to minimise steric interactions between the protein and the enzyme. Enzyme activity is thus restored and can be measured by the fluorescence emission of fluorescein **19** produced from the enzyme-catalysed hydrolysis of non-fluorescent substrate, fluorescein diacetate (FLDA) **18**. The use of an enzyme for signal amplification was found to increase fluorescence signals 70-fold. This versatile approach was demonstrated for the detection of a number of different proteins through simple alteration of the inhibitor tag.



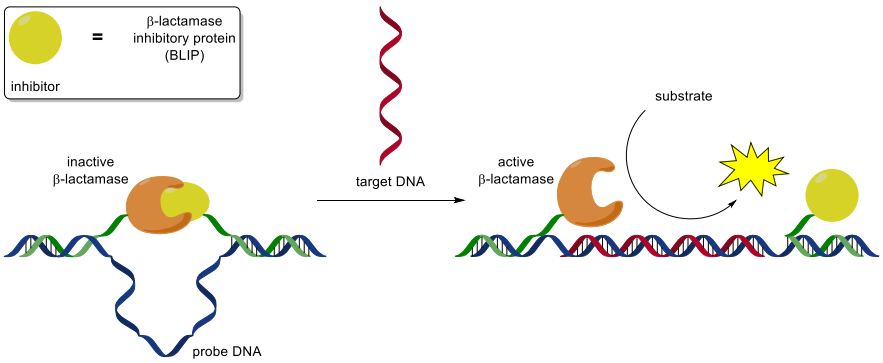
Scheme 22 Protein detection via intrasteric enzyme activation through electrostatic interactions.

Another method towards intrasteric enzyme activation for protein detection has been demonstrated by Rotello *et al*. who developed a procedure known as enzyme-amplified array sensing (EAAS) (Scheme 22).[151] In this method gold nanoparticles deliberately developed to possess a high cationic surface charge,[152] electrostatically bind to enzymes such as β-galactosidase (β-gal) that have a high anionic surface charge, and inhibit enzyme activity without denaturing the enzyme.[153] In the presence of specific proteins, the nanoparticles blocking the active site of the enzyme are removed, which restores enzyme activity. The addition of pro-fluorogenic substrate, 4-methylumbelliferyl-β-D-galactopyranoside (4-MUG) **20**, allows for protein detection *via* fluorescence. The amplification provided by the enzyme delivers a highly sensitive detection assay able to differentiate target proteins at 1 nM concentrations. The versatility of using enzymes as amplifiers under physiological conditions is also demonstrated as using an alternative enzyme substrate enabled a colourimetric biosensor for bacterial infections to be developed.[154] Furthermore, this electrostatic nanoparticle approach to protein detection can be used in conjunction with fluorescent conjugated polymers, *vide infra*, to develop 'chemical nose' sensors capable of distinguishing between several different proteins simultaneously.[155]

Enzymes have also been used in a number of amplified nucleic acid detection methodologies due their ease of conjugation to DNA as well as rapid signal production.[156] One of the first examples of such a system was described by Ghadiri *et al.* who developed a modified protease enzyme where its phosphoramidate inhibitor was covalently bound to itself through a single-stranded DNA probe (Scheme 23).[157] Once constructed, the modified enzyme was rendered inactive since the flexibility of the DNA probe allowed for the inhibitor to loop around and bind to the active site of the enzyme. In the presence of target DNA, hybridisation occurs forming a highly favourable rigid DNA duplex and in the process, physically removes the inhibitor from the active site of the enzyme. Enzyme activity is therefore restored and hydrolysis of a non-fluorescent substrate to a fluorescent product enables simple enzyme-amplified DNA detection *via* fluorescence. Both the sensitivity and selectivity of the concept was validated as a 10 pM concentration of DNA could be detected within 3 minutes and no discernible signal was observed in the presence of non-complementary DNA. Additionally, this approach is not limited to



Scheme 23 DNA detection via allosteric enzyme activation through intrasteric enzyme regulation.

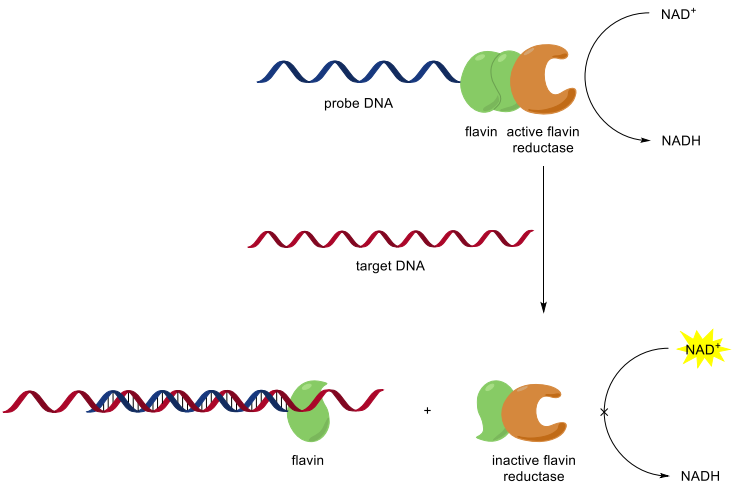


Scheme 24 Mechanical intrasteric enzyme activation for DNA detection.

the use of proteases for signal amplification as the enzyme thrombin has also been conjugated to an inhibitor–DNA probe for fluorescent DNA detection.[158] The excellent control of enzyme activity demonstrated through allosteric regulation allows for molecular logic gates to be developed, operated by different DNA sequences.[159] Enzyme and inhibitor do not necessarily have to be covalently bound at either end of the same single-stranded DNA probe in order to achieve successful intrasteric enzyme activation as Merkx *et al.* have very recently demonstrated (Scheme 24).[160] In this approach, β-lactamase and a β-lactamase inhibitory protein (BLIP) were attached to separate strands of DNA that were complementary to that of the probe strand. Through a strong hybridisation interaction and the flexibility of the single-stranded probe strand, enzyme and inhibitor are brought together restricting substrate turnover. In the presence of target DNA, hybridisation with the probe strands causes significant structural rigidity and physically pulls the inhibitory protein from the enzyme active site and away from one another, thus restoring enzyme activity. Again, this can be measured by fluorescence through the introduction of a non-fluorescent enzyme substrate to afford the fluorescent product. This modular approach to intrasteric enzyme activation for sensitive DNA detection could detect down to 2 fmol of target single-stranded DNA.

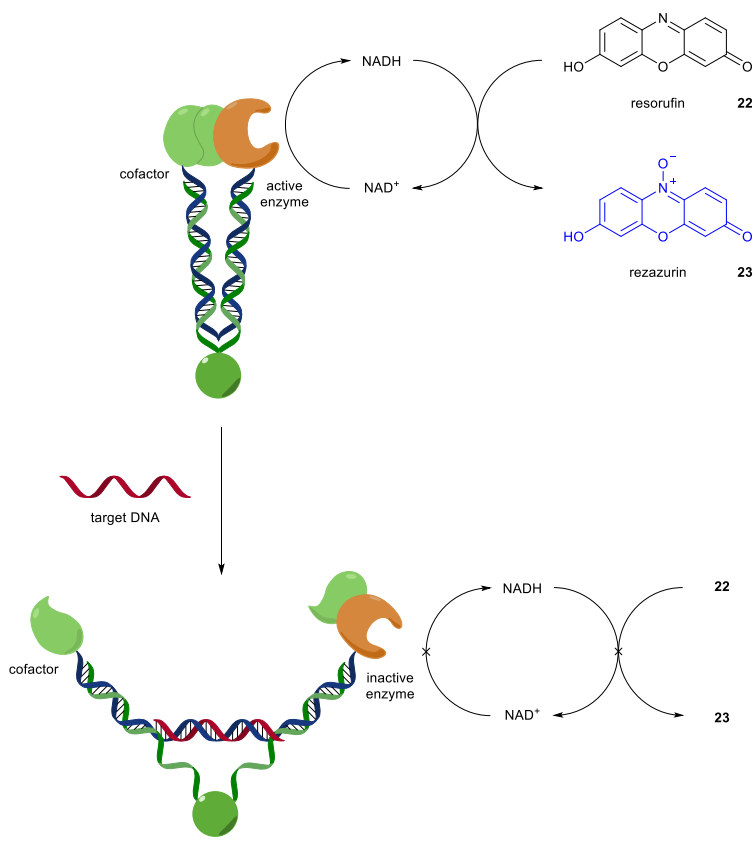
Enzyme activity can also be regulated through selective control of specific allosteric factors. In principle, allosteric activation involves target analyte recognition at an allosteric binding site which, in turn, causes a structural conformation that activates the catalyst. The excellent versatility of being able to regulate enzyme activity through allosteric activation, rather than intrasteric activation, has seen this method used extensively for signal amplification within biosensors for molecular diagnosis.[161] Allosteric enzyme activation methods can vary tremendously, but generally either occurs through manipulation of enzyme cofactors or through modification of the enzyme structure. Enzymes are usually dependent upon cofactors, such as small organic molecules,[162] or metal ions,[163] for high catalytic activity. Therefore, a popular allosteric enzyme activation method for sensing is to release enzyme cofactors selectively through an analyte–probe interaction at an allosteric binding site to consequently up-regulate enzyme activity.

Through the manipulation of flavin, a small organic molecule cofactor, Décout *et al.* have also developed an allosteric enzyme activation method for fluorescent DNA detection (Scheme 25).[164] Modifying flavin with a single-stranded DNA probe was shown not to significantly interfere with its ability to act as a cofactor, thus enzyme activity remained on consuming fluorescent substrate, nicotinamide adenine dinucleotide (NAD^+). In the presence of target DNA, the rigid DNA double helix formed as a result physically removes the cofactor from the allosteric site of the enzyme, which down-regulates enzyme activity. As such, NAD^+ does not get consumed by the enzyme which means fluorescence remains on. This approach to DNA detection is reported to detect a 50 nM concentration of target DNA after a 2 hour reaction time. Although no optimisation was performed to try to increase the sensitivity of this method, inherent drawbacks to this system include a lower affinity of the modified flavin towards the enzyme and the weak fluorescence of NAD^+.



Scheme 25 Fluorescent DNA detection via allosteric organic cofactor release and consequential down-regulation of enzyme activity.

A similar approach has been demonstrated by Yan *et al.* who use molecular tweezers for enzyme-amplified DNA detection (Scheme 26).[165] Molecular tweezers are synthetic molecular receptors with two interaction sites bridged by a hinged spacer.[166] For the detection of DNA, the allosteric site in this example was constructed of single-stranded DNA complementary to the target, whilst enzyme and cofactor were attached to either end of the tweezer arms. In the absence of any

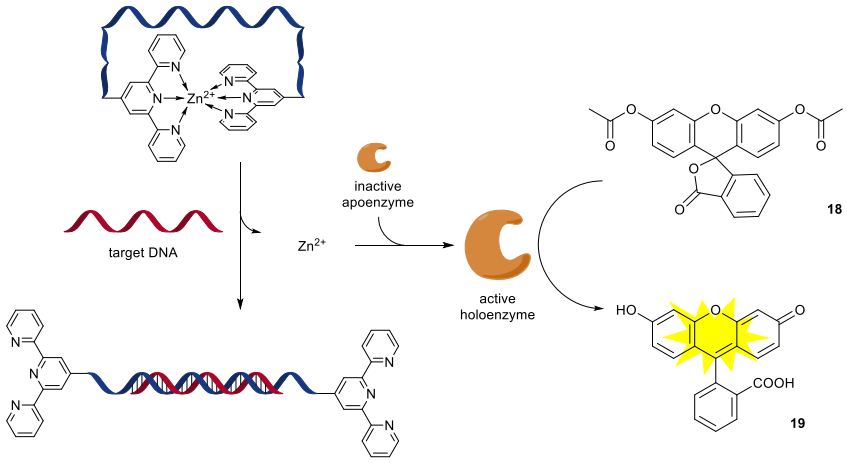


Scheme 26 Molecular tweezer approach to colourimetric DNA detection via allosteric enzyme activation.

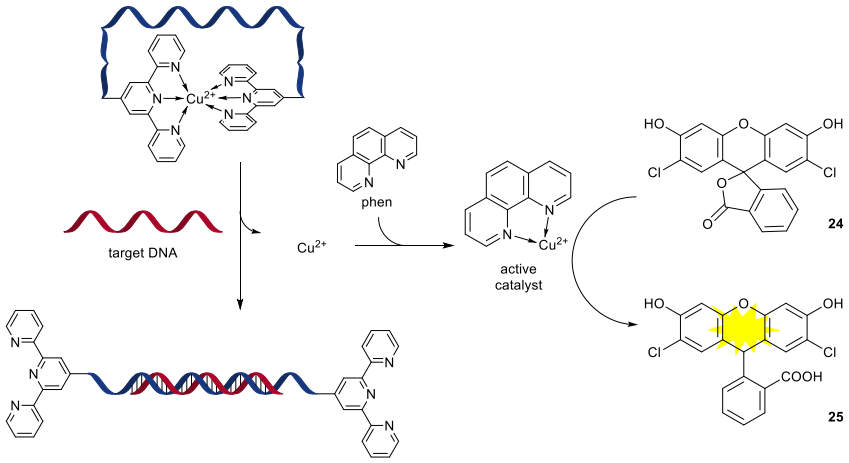
target DNA, the tweezers are closed bringing enzyme and cofactor together and activating enzyme activity. However, in the presence of target DNA, a rigid double helix is formed within the tweezers which prizes enzyme and cofactor apart, deactivating enzyme activity. By coupling enzyme activity with the production of blue dye resazurin **23**, which produces a greater signal than using NAD^+ alone, 0.75 µM target DNA could be indicated through the absence of colour production.

Metal ion cofactor release has also been exploited for allosteric enzyme activation for sensing and has been demonstrated effectively by Graf and Krämer for fluorescent DNA detection (Scheme 27).[167] In the presence of Zn^{2+}, single-stranded DNA, complementary to that of the target, modified at each end with a terpyridine moiety forms a stable cyclic structure.[168] Upon the addition of target single-stranded DNA, a conformational change occurs to accommodate the formation of the more favourable DNA double helix and bis-chelation to the metal ion is disrupted. The inactive apoenzyme of carbonic anhydrase (CA) then binds to the released metal to form the active holoenzyme. Enzyme activity, which indicates the presence of DNA, can be detected by the deacetylation of FLDA **18** to give the fluorescent product fluorescein **19**. Despite this elegant approach towards allosteric enzyme activation, a significant background reaction by the apoenzyme limits the sensitivity of the assay to target DNA concentrations of only 0.1 µM. This high background reaction observed with CA was considerably reduced by replacing the enzyme with a zinc-dependent aldolase which, *via* a signal transduction mechanism, results in the consumption of UV-active substrate NAD^+, thus developing a switch-off DNA detection method.[169]

Although many enzymes require cofactors, releasing them in response to certain analytes still remains a substantial challenge. This approach towards allosteric enzyme activation for signal amplification relies on having a high affinity between the cofactor and enzyme as well as a low background rate of the apoenzyme in the absence of the cofactor. However, as shown previously, these criteria can be difficult to fulfil. Despite the advantages of using enzymes as amplifiers within sensing methodologies such as their high turnover number, they are expensive, can be difficult to manipulate without denaturising and they are unable to be used under non-physiological conditions. Because of this, synthetic catalysts have gained increased interest as amplifiers within sensing as they can be easier to manipulate, have a higher tolerance to changes in conditions and have application to the detection of a wider range of analytes. Similarly to enzyme activation, most catalytic signal amplification methodologies use either intrasteric or allosteric strategies for controlling catalytic activity.



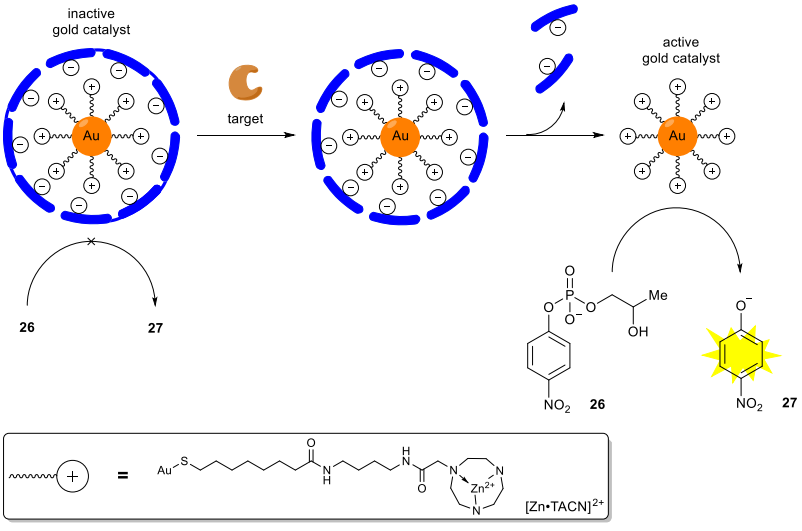
Scheme 27 Fluorescent DNA detection via allosteric metal ion cofactor release and consequential up-regulation of enzyme activity.



Scheme 28 Fluorescent DNA detection via allosteric metal ion release and catalyst activation.

Intrasteric activation of catalysts for sensing involves target recognition at the catalyst active site. In principle, catalyst activity can be significantly reduced by physically blocking the catalyst active site with functionalised molecules. If these molecules are functionalised with analyte-responsive groups, then only in the presence of the analyte should the selective removal of the catalyst inhibitor occur, which would reveal the catalyst active site and restore catalytic activity. Simply coupling catalytic activity with signal production leads to a successful analyte detection assay with catalytic amplification. Following their initial publication on the allosteric activation of enzymes for DNA detection, Krämer *et al*. adapted the same probe, and therefore the same analyte recognition procedure, for a synthetic catalyst-amplified method for DNA detection (Scheme 28).[170] Building upon the idea of DNA-templated metal catalysis,[171] the authors replaced Zn^{2+} with Cu^{2+} which, in the absence of the target, still forms the same stable cyclic structure. In the presence of the target single-stranded DNA, the formation of the double helix occurs and in the same manner as shown previously, the metal is released into the system. An active catalyst is then formed between the released metal and phenanthroline (phen), a ligand capable of accelerating copper-catalysed reactions. Non-fluorescent dichlorofluorescein (DCF) **24** is then oxidised by the active Cu·phen catalyst to give the fluorescent product dichlorodihydrofluorescein (DCFH) **25**. The low turnover rate of the active catalyst however limits DNA detection to a concentration of 5 nM.

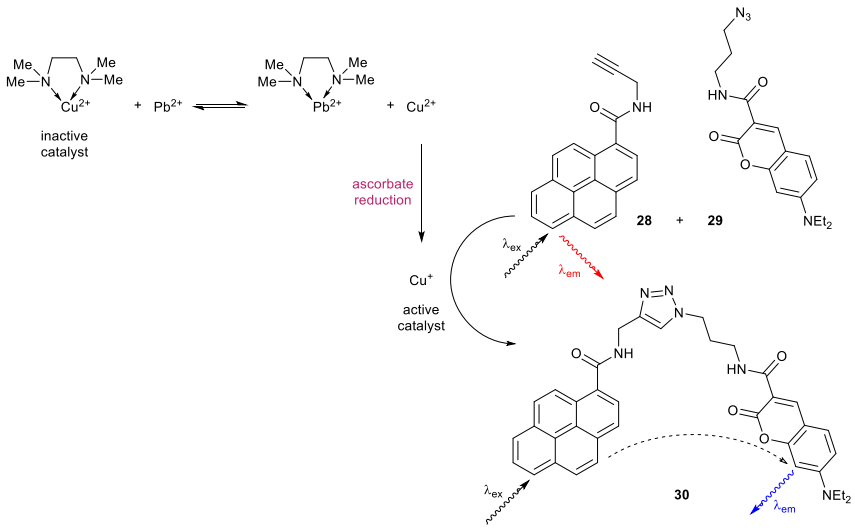
Prins *et al.* were also able to utilise intrasteric regulation of a synthetic catalyst for a colourimetric enzyme detection assay (Scheme 29).[172] In this example, gold nanoparticles, covered with triazacyclononane (TACN) ligands capable of binding Zn^{2+}, were prepared and the resulting cationic complexes were shown to effectively catalyse transphosphorylation reactions.[173] However, this catalytic activity could be switched off through the binding of oligoanions, such as peptides, to the catalyst through electrostatic interactions, which physically block the active site of the catalyst. When the target protease enzyme was added, cleavage of the peptides occurred which reduces the effectiveness of the binding between blocker and nanoparticle. Destabilisation reveals the catalyst active sites and catalyses the dephosphorylation of substrate **26**, which is accompanied by the production of yellow reporter molecule *p*-nitrophenol (PNP) **27**. The benefits of this system include a close to zero background rate, a high affinity of the $[Zn^{2+}{\cdot}TACN]^{2+}$ complexes towards the substrate and a colourimetric detection system. Also, the catalyst-amplified enzyme detection assay was shown to be highly sensitive, capable of detecting protease subtilisin A at a concentration of 66 nM after an hour reaction time.



Scheme 29 Intrasteric activation of a synthetic catalyst for catalyst-amplified colourimetric enzyme detection.

Intrasteric catalyst regulation through the use of analyte-responsive probe molecules has some inherent difficulties associated with it since the catalyst inhibitor requires strong enough binding to prevent a high background reaction but not strong enough to prevent an efficient turn on of catalytic activity in response to the analyte. As such, signal amplification by allosteric catalysis (SAAC) has by far become the most popular catalytic signal amplification strategy used for sensing.[174] Primarily, this is due to the vast number of supramolecular receptors used for analyte detection and also the large number of synthetic catalysts used for amplification that can be easily coupled together for an amplified detection system.[175] Consequently, a significant number of

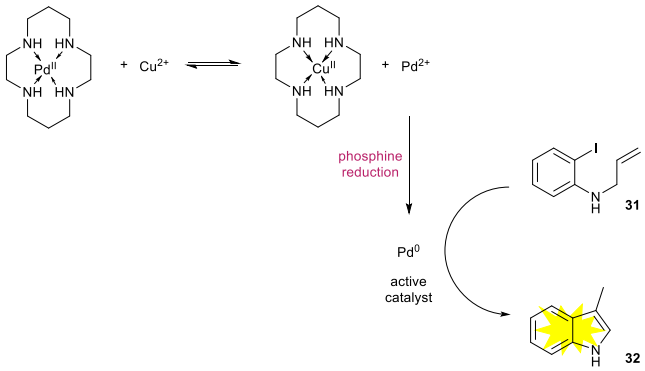
signalling techniques based upon supramolecular systems have been developed for sensitive DNA, protein and small molecule detection.[176] Additionally, the binding affinities between different metal–ligand complexes are well-known allowing for ligand exchange to become a prominent analyte detection, and subsequent catalyst activation, mechanism for SAAC.



Scheme 30 Allosteric catalyst activation for FRET-based detection of lead ions.

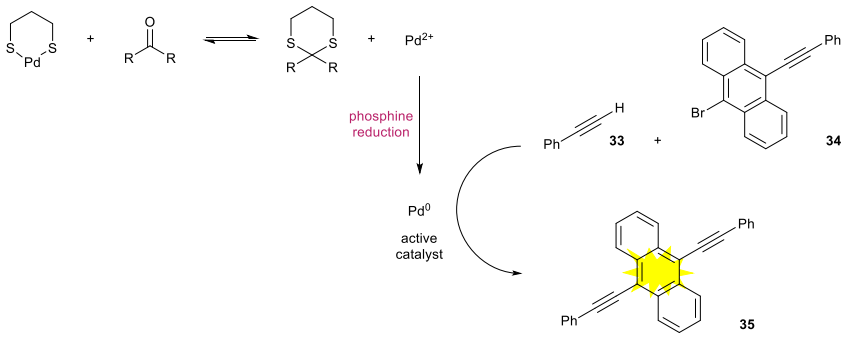
Anslyn *et al.* were the first to exploit ligand exchange for SAAC allowing for the detection of lead ions (Scheme 30).[177] In this approach, a Cu^{2+} complex with tetramethylethylenediamine (TMEDA) was synthesised and shown to be an inactive catalyst within a CuAAC reaction. Metal cations, such as Pb^{2+} and Zn^{2+}, were found to competitively coordinate with TMEDA and consequently release Cu^{2+} into the system. In the presence of L-ascorbate, Cu^{2+} is reduced to Cu^{+}, the active catalyst within the CuAAC reaction. By using FRET pair pyrene-derived alkyne **28** and coumarin-based azide **29** as the reactants, target metal ion concentration could be measured directly by fluorescence through the resulting triazole product **30**. Although being the seminal publication in the field, this approach to catalytic signal amplification is not without limitations. The analyte in question has to have orthogonal reactivity to the reaction conditions to not cause unwanted side-reactions. For example, Hg^{2+} was shown not to activate the cycloaddition reaction, but instead oxidised the reducing agent. Ligand exchange was also found to be slow, limiting the amount of active catalyst available relative to the concentration of analyte. Since the catalytic amplification is a bimolecular reaction, under the dilute assay conditions the reaction was deemed incomplete even after 18 hours, which further shows the inefficiencies in this particular catalytic system. Additionally, a high concentration of the analyte was found to interfere with the amplification reagents, potentially due to the formation of metal acetylides.

In an ensuing publication,[178] the authors sought to overcome some of these limitations, by choosing to use a more efficient palladium catalyst, as the catalytic signal amplifier (Scheme 31).§ The ligand exchange strategy, which allows analyte detection through allosteric regulation, was improved by replacing the relatively unselective TMEDA ligand with a polyazacyclam (PAC), which provided better selectivity for Cu^{2+} over other metals. Also, by designing a catalytic signal amplification methodology around a palladium catalyst, an intramolecular Pd^{0}-catalysed Heck reaction could be used, which provides increased ease of operation and the formation of a fluorescent product.[179]



Scheme 31 Allosteric catalyst activation for the fluorescent detection of copper ions.

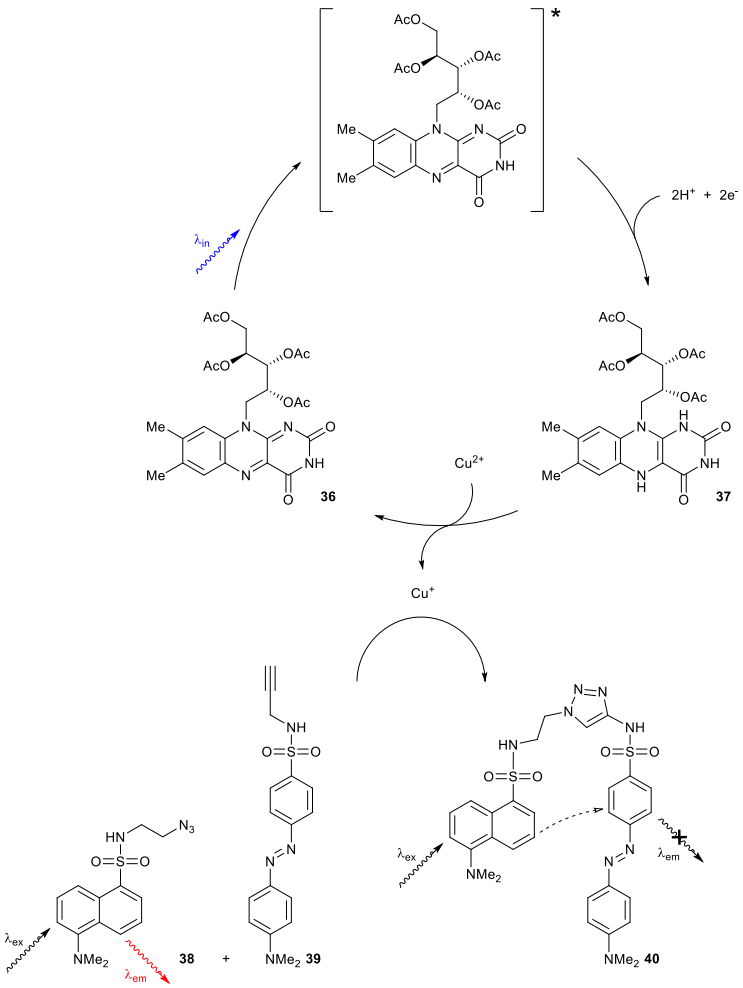
In this strategy, a Pd·PAC complex was synthesised that was shown to be inactive towards the Heck reaction. When the metal ion analyte is present, in this case Cu^{2+}, ligand exchange occurs since Cu^{2+} has a higher affinity for the PAC than Pd^{2+}, releasing Pd^{2+} into the system which is immediately reduced to the active Pd^{0} by a phosphine ligand. The resulting active catalyst is able to turn non-fluorescent aryl iodide **31** into fluorescent indole **32** allowing for catalyst activity and therefore analyte concentration to be detected *via* fluorescence. Increasing catalytic activity improved the catalytic signal amplification as a detectable fluorescent signal was provided from a 30 nM concentration of Cu^{2+}. Despite this, a 90 minute reaction time was required and the ligand exchange methodology for analyte detection was shown to be unselective, since Ni^{2+}, Co^{2+} and Cd^{2+} all delivered false positive results. Better selectivity for the toxic metal ion Cd^{2+} over Ni^{2+} and Co^{2+} could be achieved by using a PAC with a bigger ring size to accommodate for the increase in ionic radius.[180] Better sensitivity was also achieved through the production of a coumarin-derived product since the presence of an electron-withdrawing ester not only increases the rate of Heck coupling but the product also has an increased quantum yield than that of indole **32**.



Scheme 32 Allosteric catalyst activation for the fluorescent detection of small, carbonyl-containing organic molecules.

The use of PACs for analyte recognition is a major drawback for this methodology since analyte detection is limited to only metal ions. Also, ligand exchange is slow, reversible and as shown previously, unselective. To extend the application of this catalytic signal amplification methodology beyond the detection of metal ions, the group replaced the PAC with a dithiol for the fluorescent detection of carbonyl-containing small organic molecules (Scheme 32).[181] In this approach, target compounds containing carbonyl functionality, such as a ketone or aldehyde, condense with the analyte-recognising dithiol to give a dithiolane, which has a considerably lower binding affinity towards metals than the free thiol. As a result, Pd^{2+} ions are released into the system and are reduced by phosphine ligands present in the assay mixture, generating the active catalyst for a Sonogashira cross-coupling reaction.[182] The coupling of phenylacetylene **33** with 9-bromo-10-(phenylethynyl)anthracene **34** produces the commonly used fluorophore 9,10-bis(phenylethynyl)anthracene (BPEA) **35**, therefore allowing analyte detection to be easily detected through fluorescence. Despite the catalytic signal amplification, significant drawbacks with using the Sonogashira cross-coupling reaction for catalytic signal amplification is its intolerance of water and some of the reaction components cause undesired quenching of anthracene fluorescence. However, the initial work published by the Anslyn group proved the concept of SAAC and has paved the way for this design to be adapted within other signal amplification methodologies that utilise allosteric control of metal ions for catalyst activation.

One such methodology was described by Ritter and König who developed a signal transduction and signal amplification protocol using photo-activated catalysis.[183] In this procedure, a flavin derivative is used as a synthetic photoreceptor while the CuAAC reaction is used for signal amplification (Scheme 33). Specifically, the stimulation of riboflavin tetraacetate **36** by a photon gives its strongly-oxidising excited state which, in the presence of an electron donor, undergoes photoreduction to afford strongly-reducing dihydroflavin tetraacetate **37**. Dihydroflavin **37** is then capable of reducing Cu^{2+} to Cu^{+}, thus providing the active catalyst for the cycloaddition, resulting in the formation of triazole **40** from azide **38** and alkyne **39**. Signal detection was measured through the reduction of observed fluorescence since the covalent connection between fluorophore and quencher reduces fluorescence emission.[184] In water, the reaction cascade shows light-dependent behaviour as only under irradiation is significant fluorescence quenching observed. When irradiation is stopped, disproportionation of Cu^{+} to Cu^{0} and Cu^{2+} occurs, which removes the active catalyst from the system and stopping the cycloaddition reaction. However, it was found that excess continuous irradiation leads to the formation of undesired by-products. Other problems associated with the assay include requiring oxygen-free conditions to prevent formation of flavin hydroperoxides and a slow background reaction in the absence of light due to spontaneous reduction of Cu^{2+} to Cu^{+} by triethylamine in solution. However, the principle of transforming a non-chemical input signal, in this case

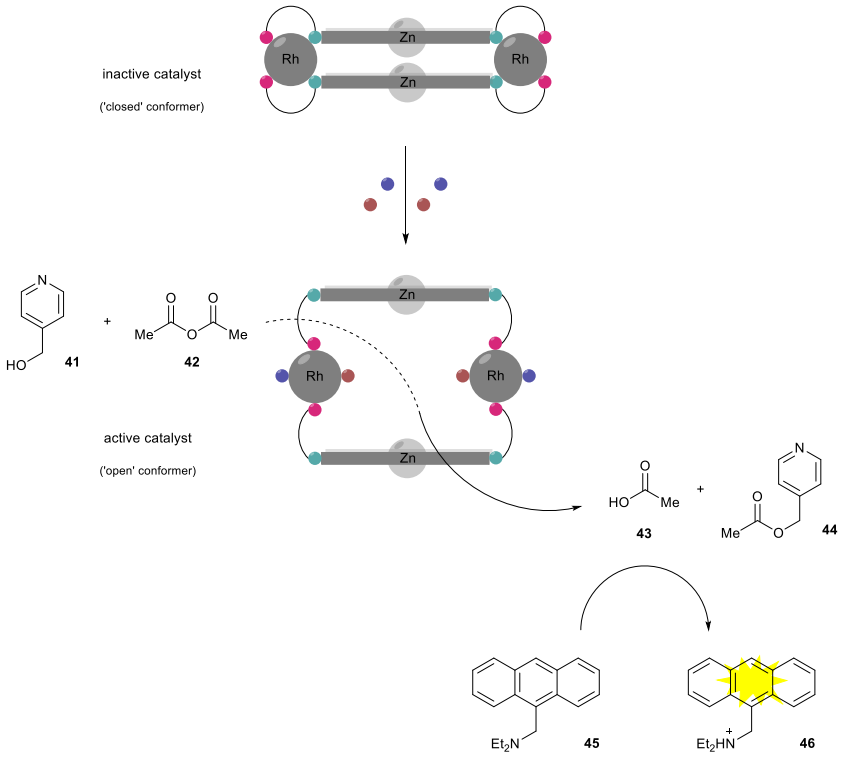


Scheme 33 Light-activated catalytic signal amplification.

light, into an amplified chemical output signal is superbly illustrated in this example, inspiring other groups to develop similar signal transduction and amplification chemical systems.

The majority of examples of allosteric catalyst activation previously described have been based upon metal–ligand binding affinities where, analyte recognition leads to coordination disruption followed by metal ion release and consequent active catalyst formation. However, this is an imperfect approach since analyte recognition is a reversible reaction between the analyte and the active catalyst responsible for signal amplification. Also, the formation of the active catalyst is not necessarily instantaneous after analyte recognition which leads to long assay times. To overcome these problems, Mirkin *et al.* have developed synthetic enzyme mimics based upon supramolecular coordination chemistry.[185]

To achieve this, inorganic macrocycles were constructed *via* the weak-link approach (WLA) consisting of two metal centres and two hemilabile ligands.[186] The ligands are the key to this concept as they are polydentate chelates containing two sets of two differently coordinating heteroatoms; one set that strongly binds to the metal centre and one set that weakly binds to the metal centre. The dimer that initially forms has all coordinating atoms bound to a metal centre and in this 'closed' structural conformation, the ligand spacers are forced together. The introduction of small, coordinating molecules, that have a higher affinity to the metal than the weakest metal–heteroatom bond, displace the weakly binding heteroatom of the ligand. This then causes a structural conformational change to give an 'open' complex since the ligand spacers are now pulled apart. This precise control over the structural conformation of a macrocycle led to the subsequent development of a synthetic supramolecular catalyst where catalyst activity could be regulated allosterically.[187]



Scheme 34 Allosteric regulation of a supramolecular catalyst for catalytic signal amplification.

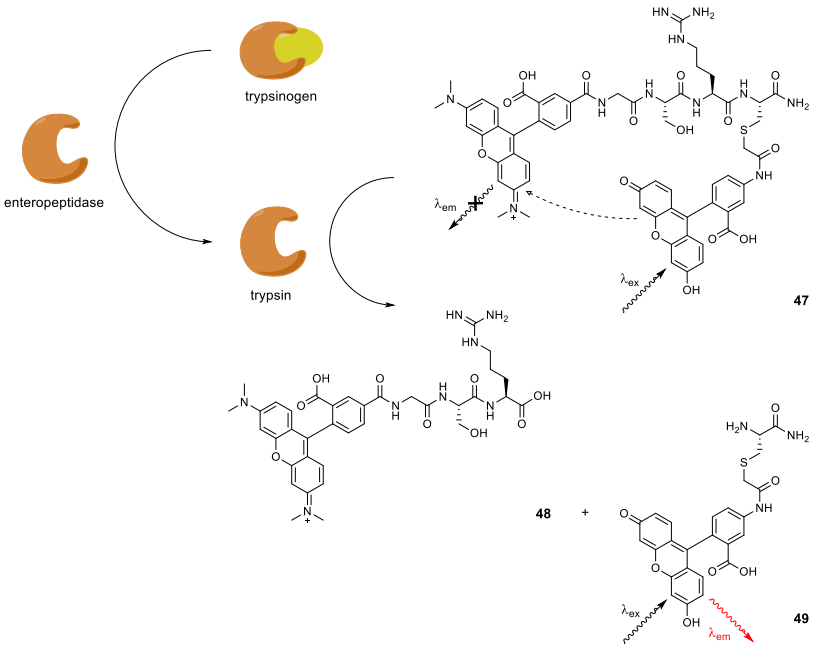
To obtain catalytic activity selectively as a result of a structural change in conformation, the ligand spacers were replaced with a $[Cr{\cdot}salen]^{3+}$ complex proven to catalyse epoxide ring-opening reactions.[188] In the absence of the small molecule effectors, the tetrametallic supramolecular construct was shown to be in its 'closed' conformation and exhibited minimal catalytic activity towards the ring-opening of cyclohexene oxide since the catalyst active sites are pushed too close together and thus blocking one another, preventing the substrate from reaching the catalyst active site. The addition of small molecule effectors Cl^- and CO result in them binding to the allosteric Rh^+ metal centre and causing a structural change in the macrocycle to its 'open' conformer, known as halide-induced ligand rearrangement (HILR).[189] The Cr^{3+} metal centres are then pulled apart from one another revealing the active sites for the substrate, which consequently leads to a 25-fold increase in catalytic activity. In addition, the bimetallic active site of the supramolecular catalyst was shown to have an increased rate of reaction over its monomeric counterpart, which explains the low background rate observed when the catalyst is 'closed'.[190] Since the epoxide ring-opening reaction is not attributed with a change in physical property, signal amplification *via* this approach to allosterically-regulated supramolecular catalysis could not be used for sensing purposes.

Towards this end however, the group sought to change the catalyst responsible for signal amplification to a $[Zn{\cdot}salen]^{2+}$ complex capable of catalysing the acylation of 4-pyridyl carbinol **41** (Scheme 34).[191] As a consequence of the acylation, the pH of the reaction medium is lowered since acetic acid is formed as a by-product and as such, signal production was achieved through a pH-responsive fluorophore **45** allowing for analyte detection to be determined using fluorescence.[192] Under an atmosphere of carbon monoxide, an 800 nM concentration of Cl^- ions could be distinguished from the background reaction after 6 hours. Since the supramolecular catalyst is modular by design, alternative metals can be used at the allosteric site of the supramolecular catalyst yet still maintain the same mode of activation and the same catalytic reaction for amplification. This can therefore expand the application of the signal amplification procedure towards the detection of different analytes.[193] It is thought that increased control and a reduction in background catalytic activity could be achieved through a triple-layer catalyst where the middle layer holds the

active catalyst and the top and bottom layers physically block the catalyst active sites when the supramolecular structure is 'closed'.[194] Currently, the addition of the small molecule allosteric effectors can induce a structural change in the supramolecular structure to its 'open' conformer and restore catalytic activity to polymerise ε-caprolactone,[195] but has yet to be applied towards a sensing methodology.

Double-Catalyst Signal Amplification

In principle, the addition of an extra catalytic cycle to a single-catalyst signal amplification procedure should accelerate the rate of signal production over time and in turn, would lead to a signal increase orders of magnitude higher than that of just doubling the number of catalysts of a single-catalyst system. However, in comparison to the number of single-catalyst signal amplification procedures described, the use of two sequentially-activated catalysts for signal amplification is a considerably less explored area. This could be, in part, due to the difficulty in finding catalyst combinations whereby products resulting from the first catalytic cycle are able to act as catalyst activators for the second catalytic cycle. In addition, to afford sufficient assay sensitivity, the background reaction of the second catalyst must be minimal, if not zero, to minimise false positive results. Initially, signal amplification protocols utilising a two catalyst cascade were developed as a result of applying a single catalytic amplification procedure towards the detection of a target that itself also displays catalytic activity.



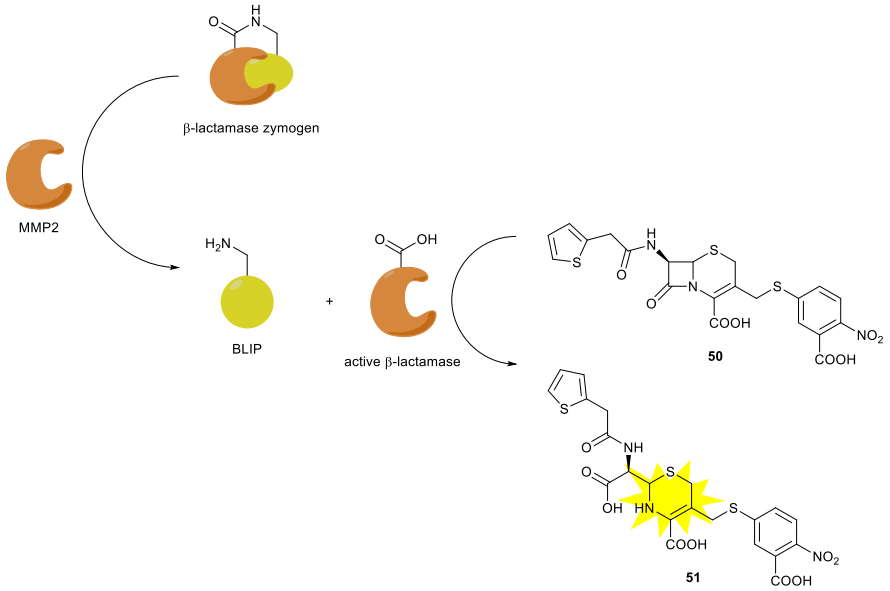
Scheme 35 Double-catalyst signal amplification approach to the FRET-based detection of enteropeptidase.

The first example of a double catalyst signal amplification strategy for a sensing application was demonstrated by Zuchner *et al.* for protease detection using FRET (Scheme 35).[196] Sensitive protease detection is of paramount importance as abnormal protease activity has been associated with a variety of diseases, one of which includes cancer.[197] As such, there has been huge demand for the development of highly sensitive protease assays and methods to achieve high sensitivity have been through the use of enzyme cascades for signal amplification.[198]

In this particular example, an initial optimisation of a FRET-based detection assay was performed on trypsin, a digestive enzyme that selectively cleaves peptides at the carboxyl side of amino acids lysine or arginine.[199] Of the trypsin substrates synthesised and tested, peptide **47** was found to be optimal as it showed a >97% quenching efficiency in the absence of the enzyme and after trypsin was introduced, maximum fluorescence was obtained in 15 minutes, achieving a LOD of 0.1 fmol. With the second catalytic cycle of the amplification methodology optimised, the next objective was to selectively activate it.

To do this, the group chose serine protease enteropeptidase, the intestinal enzyme responsible for activating multiple digestive enzymes in response to food ingestion,[200] and whose congenital deficiency can lead to severe intestinal malabsorption.[201] Its primary mode of action is to catalyse the activation of trypsin from the inactive zymogen, trypsinogen.[202] As such, trypsin was replaced with trypsinogen and exposed to enteropeptidase to determine if the enteropeptidase could catalyse the activation of the second catalytic signal amplification. Indeed, in the presence of enteropeptidase, a fluorescent signal was obtained which was attributed to the *in situ* conversion of trypsinogen to trypsin and consequent consumption of pro-fluorescent substrate **47**. This double-catalytic signal amplification methodology achieved a LOD of 0.01 fmol for enteropeptidase after an assay time of 3 hours and demonstrated an excellent dynamic range showing a linear signal–concentration correlation over two orders of magnitude.

The high sensitivity and selectivity obtained for this dual-enzyme signal amplification approach has inspired others to do the same by developing inactive zymogens as enzyme precursors that can be activated by a clinically-relevant enzyme to produce a detectable signal. One such example can be provided by Yoo *et al.* who engineered β-lactamase zymogens for amplification and determination of protease activity (Scheme 36).[203] In this approach, an inactive β-lactamase zymogen was constructed using intrasteric inhibitor BLIP attached to the enzyme through a peptide linkage. In the presence of the protease, the peptide linkage is cleaved and intrasteric activation of β-lactamase occurs as BLIP is removed from the active site. β-Lactamase activity, and therefore protease activity, was measured by the change in absorbance from β-lactamase substrate, 7-(2-thienylacetamido)-3-((3-carboxy-4-nitrophenyl)thiomethyl)-cephalosporonic acid (CENTA) **50** to hydrolysed product **51**.[204] To prevent the BLIP from remaining

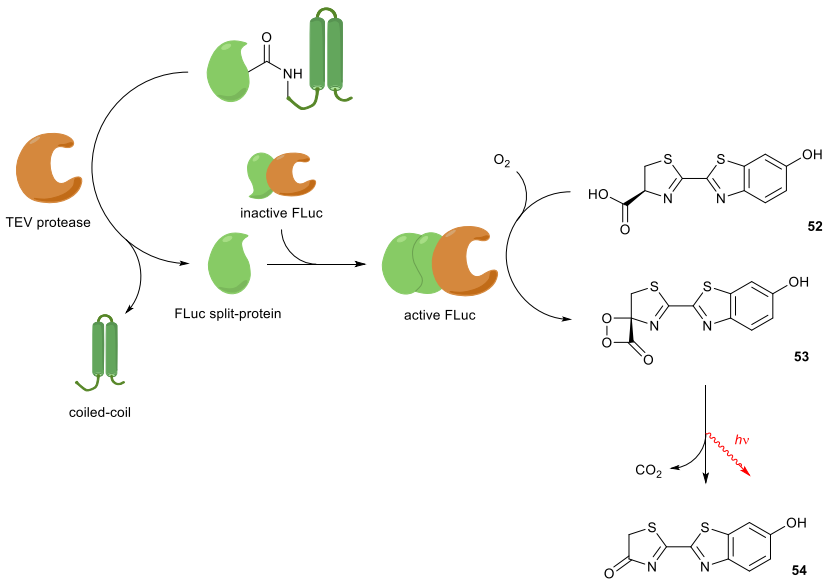


Scheme 36 Double-catalyst signal amplification for an absorbance-based protease detection assay.

bound to the β-lactamase after protease-catalysed amide hydrolysis, which would reduce the potential positive output signal, a number of β-lactamase mutants were synthesised and tested within the assay. Through this optimisation, a β-lactamase mutant with a lower binding affinity to BLIP was able to deliver a 68-fold signal increase from the background reaction. This mutant was then utilised as the signal-amplifying enzyme within a dual-enzyme signal amplification strategy for the detection of matrix metalloproteinase-2 (MMP2), a protease that plays a significant role within cancer progression,[205] and was shown to be detectable at concentrations down to 25 pM.

Another approach to enzyme-triggered enzyme activation has been through the use of split-protein systems. Split proteins are two protein fragments that do not exhibit any catalytic activity separately but can come together to form an active enzyme.[206] By physically preventing the split-proteins from coming together through an enzyme-cleavable intrasteric inhibitor, an enzyme-amplified enzyme detection assay can be achieved. This tactic was exemplified by Ghosh *et al.* who utilised the split-protein system of the enzyme firefly luciferase (FLuc) for protease detection (Scheme 37).[207] To prevent the split-proteins from coming together, one of the split-proteins was appended with a structure comprised of two interlocking α-helices known as a coiled-coil.[208–209] In the presence of the target protease, the peptide linkage between the protein and the coiled-coil is cleaved allowing the two protein halves to reassemble to form the active FLuc. Once activated, FLuc is able to catalyse the bioluminescent oxygenation of **52**, allowing protease activity to be determined *via* luminescence. Through this dual-enzyme amplification cascade, a 1000-fold increase in the signal-to-noise ratio could be obtained for the detection of tobacco etch virus (TEV) protease. The modularity of this method has allowed other split-protein type enzymes, such as β-lactamase, to be used as the enzyme amplifier and this signal amplification method has also been applied to the detection of caspases by simply changing the enzyme-cleavable linkage between the coiled-coil and the split-protein.[210]

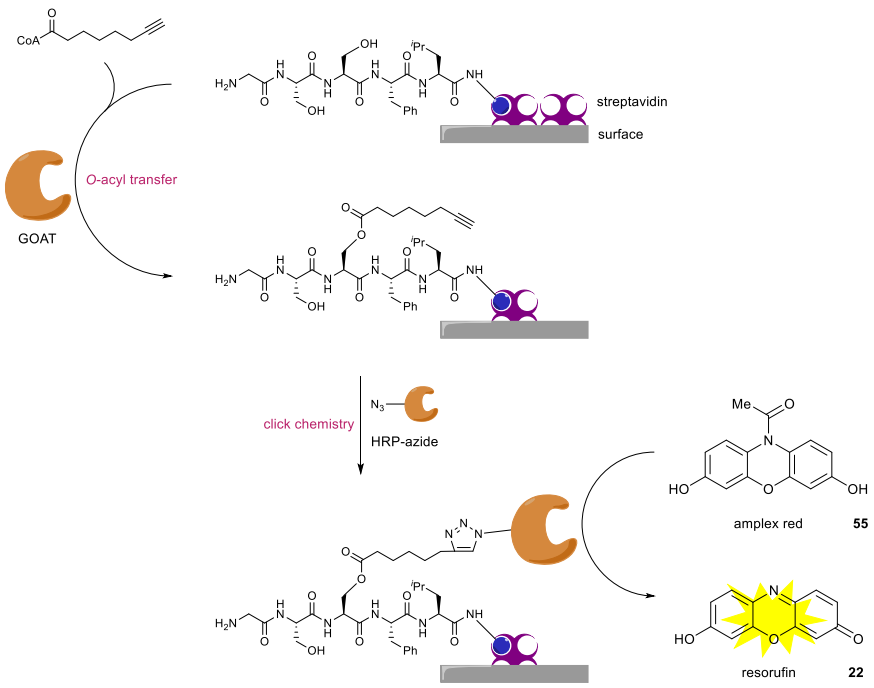
Utilising enzyme cascades for signal amplification relies on the ability to structurally modify enzymes to restrict enzyme activity without denaturisation yet in the presence of the target, enzyme activity must be easily restored. Although a number of examples have been demonstrated, this still remains a significant challenge. A way of incorporating enzyme cascades for signal amplification without needing structural modification of enzymes is to use solid-supported enzyme substrates, as enzyme activity can be introduced and removed through physical addition and aspiration steps. Inspired by assays where catalyst activity can be measured through immunoassays,[211–212] Garner and Janda developed a catalytic assay using an enzyme-linked click-chemistry assay (cat-ELCCA) for the fluorescent detection of ghrelin *O*-acyltransferase (GOAT) (Scheme 38).[213] In this approach, a biotin-labelled peptide enzyme substrate is captured on a streptavidin-coated solid surface. In the presence of the target enzyme, a selective *O*-acyl transfer occurs where



Scheme 37 Double-catalyst signal amplification for a bioluminescent protease detection assay.

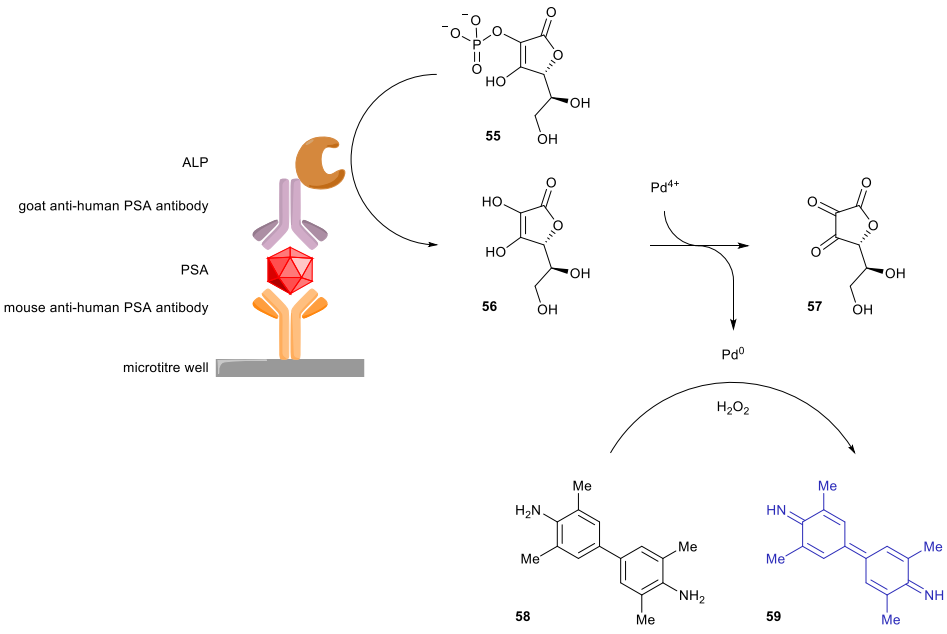
a serine residue is esterified with an *n*-octynoyl group. The resulting alkynylated product is then labelled with HRP *via* a click reaction, which is able to provide signal amplification through catalytic production of fluorescent compound resorufin **14** from HRP-substrate amplex red **47**. Through this unique double-enzyme signal amplification approach, a greater than 7-fold fluorescence enhancement was observed. Since GOAT is involved in the acylation of the gastric peptide hormone ghrelin,[214] this signal amplification methodology was subsequently used in the discovery of potent GOAT inhibitors as promising anti-obesity and anti-diabetes drug targets.[215]

However, double catalyst signal amplification is not just limited to the use of enzymes for amplification. Until quite recently, the successful application of a double-catalyst signal amplification approach towards sensing had only been achieved through a dual enzyme strategy. A methodology that utilised a hybrid enzyme–catalyst approach was first described by Tang *et al.*, for the ultrasensitive colourimetric detection of PSA (Scheme 39).[216]



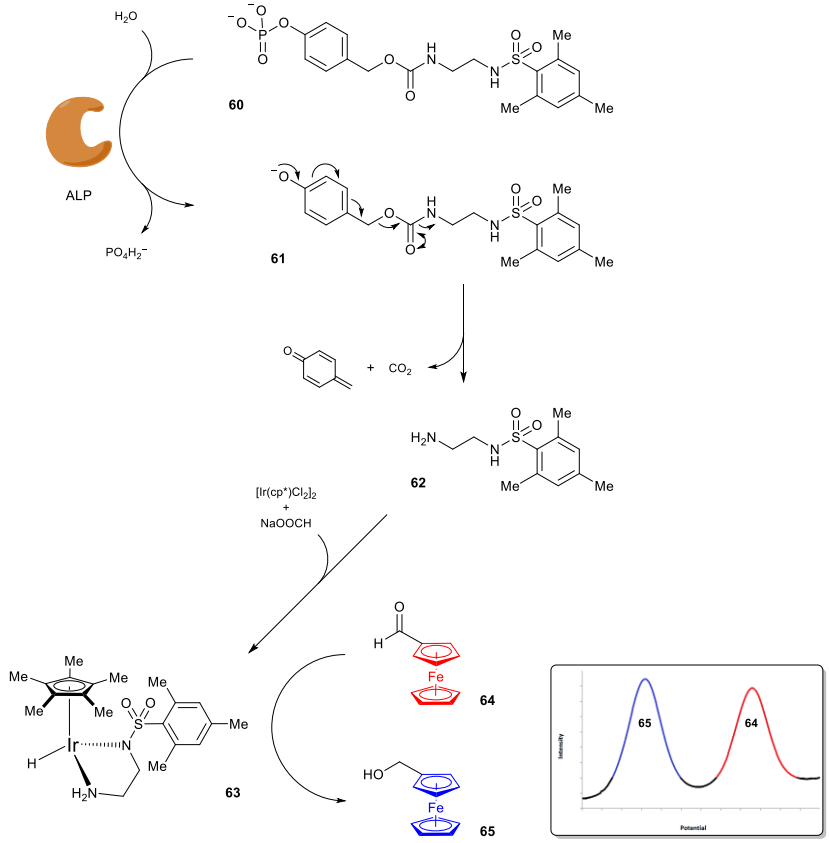
Scheme 38 Catalytic assay using enzyme-linked click-chemistry assay (cat-ELCCA) for the fluorescent detection of ghrelin O-acyltransferase (GOAT).

In this example, a typical sandwich ELISA was constructed, designed for the detection of PSA, using ALP as the enzyme label. In the presence of the enzyme, and therefore the target, ALP substrate L-ascorbic acid 2-phosphate (AA2P) **55** becomes hydrolysed to ascorbic acid (AA) **56**. AA was then able to reduce a palladate species to the active catalytic palladium nanostructures that aggregate upon the surface of non-participating gold nanoparticles.[217] As a peroxidase mimic, the palladium nanostructures can catalyse the hydrogen peroxide-mediated oxidation of 3,3',5,5'-tetramethylbenzidene (TMB) **58** to its corresponding blue diimine **59**.[218] Through an extensive screening process, a positive colourimetric signal was shown to only be produced by the palladium nano-catalysts which themselves were only created in the presence of ALP, consequently leading to a low background signal. Importantly, the double-catalyst signal amplification procedure was shown to be significantly more sensitive than a traditional single-catalyst ELISA and could deliver an LOD of 0.05 ngmL^{-1} for the detection of PSA. However, due to the number of reagents required to acquire a positive signal, the procedure is considerably labour-intensive and requires the time-consuming preparation of gold nanoparticles.



Scheme 39 Double-catalyst signal amplification protocol for the colourimetric detection of PSA.

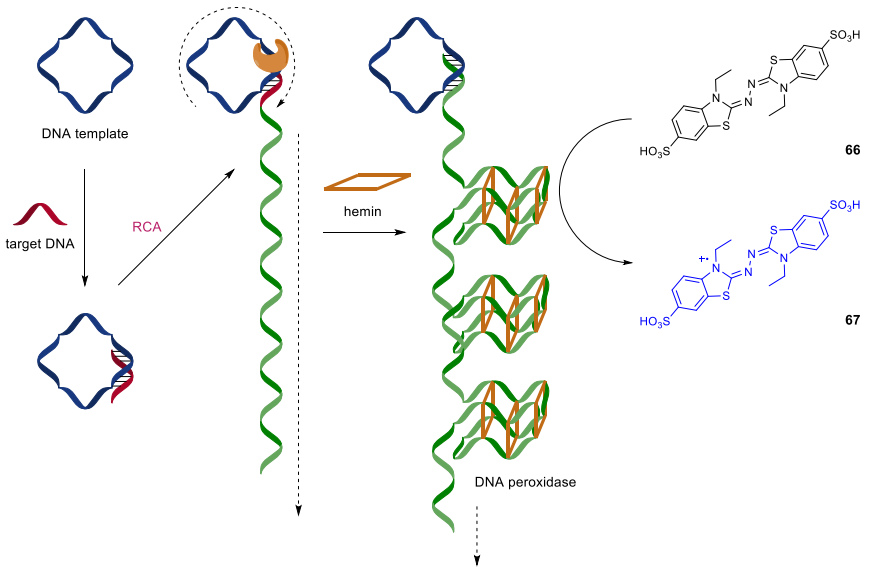
Shortly thereafter, Frost *et al.* disclosed another hybrid enzyme–catalyst double catalyst signal amplification methodology aimed at the detection of ALP (Scheme 40).[219] Rather than utilising enzyme-catalysed metal nanoparticle



Scheme 40 Double-catalyst signal amplification protocol for the ratiometric electrochemical detection of ALP.

formation for catalytic amplification, enzyme-triggered ligand release and subsequent ligand-accelerated metal catalysis was employed.[220] In this approach, enzyme substrate **60**, termed a proligand, was designed with an enzyme-responsive trigger, a self-immolative spacer and a diamine ligand. In the presence of the target enzyme, enzyme-catalysed cleavage of the trigger occurs to afford phenolate **61** which is unstable under the basic conditions of the assay and undergoes 1,6-elimination followed by decarboxylation to release ligand **62**. Once released, the ligand is capable of binding to an iridium pre-catalyst to generate active catalyst **63**, a very efficient organometallic complex within catalytic transfer hydrogenation reactions.[221] Consequently, ferrocenecarboxaldehyde **64** is reduced by the iridium catalyst to afford ferrocenemethanol **65**, both of which can be distinguished electrochemically. As a result of the powerful dual catalysis, a LOD of 7.6 pM concentration could be achieved after a 3 minute assay time. The sensitivity was compromised however due to an undesired background reaction caused by the iridium precatalyst. The protocol did exhibit excellent versatility as it was applied to a range of aldehyde catalyst substrates, easily allowing for alternate signal readout methods to be chosen if required.

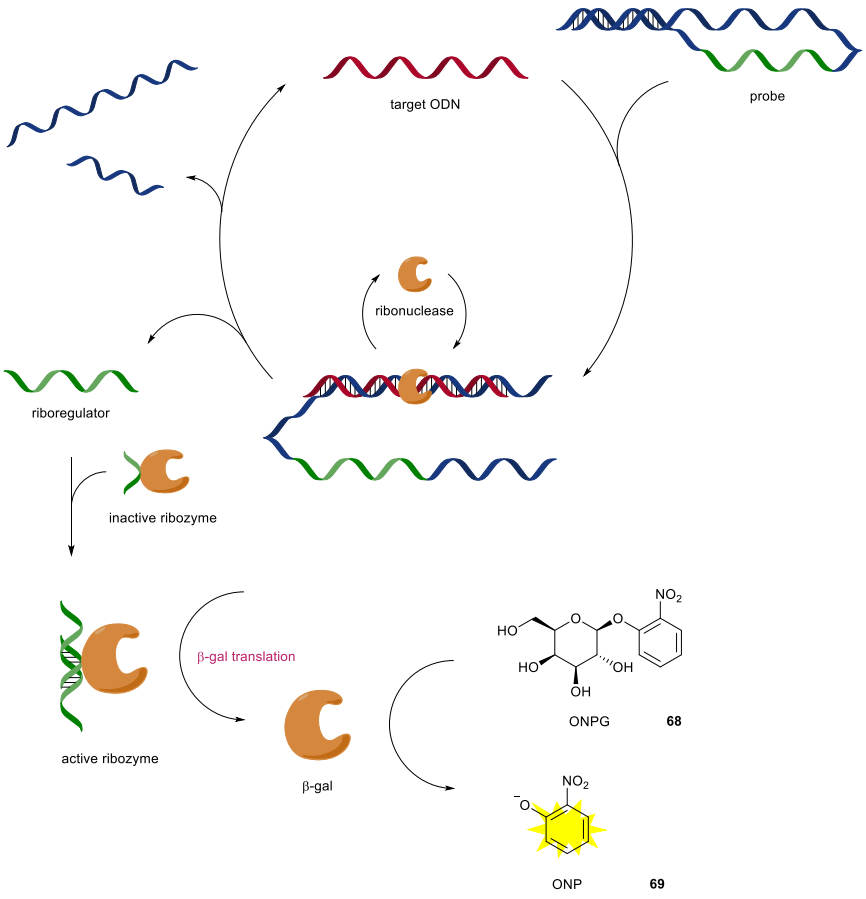
Deoxyribozymes can be used as alternatives to synthetic catalysts within a catalytic signal amplification methodology as demonstrated by Mao *et al.* who utilised a double catalyst signal amplification methodology for colourimetric DNA detection (Scheme 41).[222] Deoxyribozymes, also known as DNA enzymes or DNAzymes, are catalytic domains comprised of DNA rather than amino acids and are therefore considered to be more stable than traditional protein-based enzymes.[223] In this amplification approach, target DNA is detected using a complementary DNA probe that is also a circular template for RCA, which codes for the linear production of DNAzymes. Therefore, the target DNA acts as a primer for RCA and once hybridised, initiates the production of repeating units of DNA that form multiple DNA peroxidases after complexation to hemin. The DNA peroxidases can then catalyse the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) **66** to afford colourimetric product **67**. This procedure showed an excellent dynamic range where DNA detection covers concentrations over four orders of magnitude and delivered a LOD of 1 pM. However, problems associated with using RCA as the initial amplification procedure include strict amplification durations as any reaction time longer than optimal, results in extensive tangling of the DNAzyme chains. Not only this, but the concentration of the circular RCA template is also important as a high enough



Scheme 41 Rolling circle amplification and DNAzyme catalytic cascade for colourimetric DNA detection.

concentration is required to ensure efficient binding to the target, yet too high a concentration leads to unwanted binding of the template to the amplified DNA product thus preventing further amplification and folding of the DNA into the DNAzyme. A similar DNAzyme cascade has since been described by Tan *et al.* that replaces RCA with an alternative DNAzyme to deliver a double-catalyst signal amplification strategy for colourimetric protein detection.[224]

A similar strategy has been described by Aoyama *et al.* who use ribosomal catalytic production, or translation, of protein-based enzymes for the double-catalytic chemiluminescent detection of human immunodeficiency virus (HIV) RNA.[225] For successful RNA detection, the group utilise the molecular beacon approach,[226] where in the absence of the target, the RNA probe intramolecularly binds to itself to adopt a hairpin-like conformation which cannot activate the catalytic amplification cascade. When in the presence of the target oligodeoxynucleotide (ODN), the resulting double-stranded duplex prevents the probe from adopting the hairpin-like conformation from occurring, thus revealing the riboregulator which is the specific sequence of the probe that activates translation. Once activated, the catalytic production of luciferase enzymes begins which are able to produce a chemiluminescent output signal. By using the molecular beacon probe, excellent selectivity was achieved and due to the double-catalyst signal amplification, good sensitivity was also obtained with a target ODN concentration of 50 fmol being detectable. However, because of the size of the target–probe duplex in comparison to the riboregulator sequence, inefficient allosteric activation of the transcription enzyme was observed, leading to a loss in sensitivity.



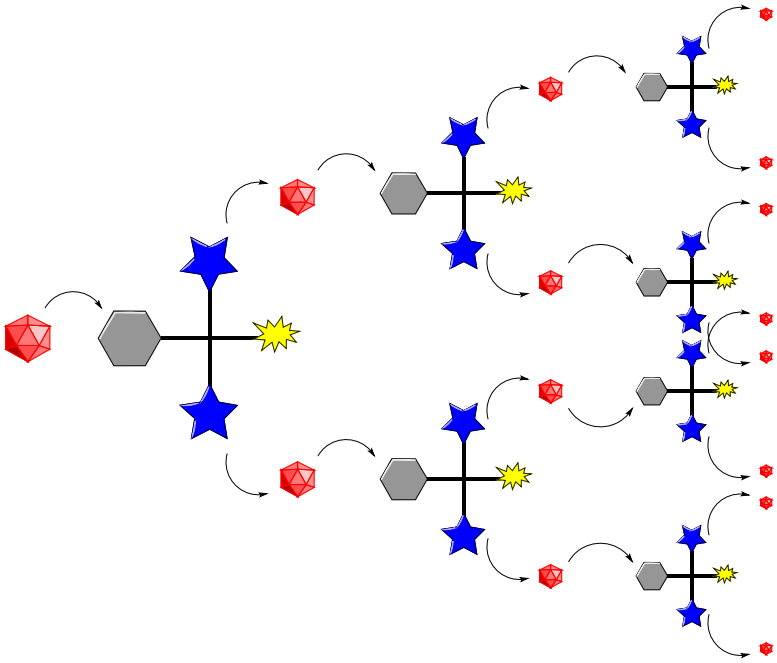
Scheme 42 Double-catalyst amplification cascade combined with EATR to obtain a triple-catalyst cascade for the visible detection of nucleic acids.

To address this, the group combined their double-catalyst signal amplification methodology with EATR to create a triple-catalyst cascade (Scheme 42).[227] In this advancement, the duplex formed as a result of target–probe hybridisation is cleaved by a ribonuclease which not only releases the riboregulator, thus making allosteric activation irreversible, but also recycles the target ODN. This both improves allosteric translation enzyme activation and allows the target ODN to react further with more probes. The extra catalytic cycle of the amplification cascade enhances both sensitivity and selectivity, obtaining a target ODN concentration LOD of 9 fmol. In addition, the riboregulator sequence can be altered to activate catalytic production of β-gal enzymes which enables the visible detection of nucleic acid sequences through conversion of *o*-nitrophenol-β-D-galactopyranoside (ONPG) **68** to *o*-nitrophenol (ONP) **69**.

The majority of double-catalyst signal amplification methodologies described to date involve the manipulation of enzyme cascades rather than the use of synthetic catalysis. This is primarily due to the well-known number and variety of enzyme cascades used in nature for a number of purposes. This leads to the easy transition from biosignalling to signal amplification within a sensing methodology by coupling the final enzyme with a colour or fluorescent production. Although selectivities and sensitivities are high, adjusting enzyme cascades towards the signal amplification of different analytes, especially when detection is required in non-aqueous solvents, can be difficult. As such, there is still demand for the easily-adaptable synthetic double-catalyst cascades for the sensitive detection of small organic molecules. Furthermore, the recent development of hybrid systems that utilise the efficiency of enzymes under physiological conditions along with the control and robustness offered by synthetic catalysts demonstrates both good sensitivity as well as excellent versatility within sensing applications.

Autoinductive Signal Amplification

Autoinduction is the process whereby the reactants or products of a reaction can directly or indirectly affect the rate of the reaction, whether it is either in a positive or negative way. This process is typically associated with metabolic enzymes in the body whereby an active pharmaceutical ingredient (API) can induce or up-regulate an enzyme involved in its own metabolism.[228] By designing a system whereby the products of a reaction can induce or up-regulate the rate of its own production, then this would provide significant signal amplification if applied to a sensing methodology. Towards this end, Sella and Shabat designed the concept of the dendritic chain reaction (DCR) (Scheme 43).[229]



Scheme 43 Amplification concept of the dendritic chain reaction (DCR).

Dendrimers themselves are uniform molecular trees made up of highly-branched, repeating units, often with reactive functionality on the end groups, which are attached to a central core.[230] Because of their unique supra- and macromolecular properties, dendrimers have found application within multiple fields such as medicine, catalysis and in particular, sensing.[231] The concept of constructing dendrimers comprised of repeating units capable of undergoing elimination mechanisms initially allowed dendrimers to be shown to selectively release their cargo in response to a specific stimulus such as, drug compounds under reductive conditions,[232] coloured compounds when exposed to palladium,[233] and fluorescent compounds in response to light.[234] The spontaneous disassembly, or self-immolation, of dendrimers in response to a specific stimulus has provided a distinctive molecular amplification approach to sensing since one analyte can trigger the disassembly of a dendrimer with multiple reporter end groups.[235] However, dendritic amplification only multiplies the initial input molecule by the number of end-groups attached to the dendrimer core. For example, in a sensing methodology for the detection of the explosive triacetone triperoxide, a dendrimer with three reporter molecules was shown to provide three times the signal compared with a non-dendritic reporter.[236] In order to provide amplification more akin to a catalyst or an enzyme in which signal amplification increases over time, DCR was designed to take advantage of the multivalent capabilities of dendrimers by attaching signal transducers as well as reporter molecules to the central core. Once released, the signal transducers could be manipulated into inducing further dendrimer disassembly, therefore leading to an autoinductive signal amplification mechanism being in effect.

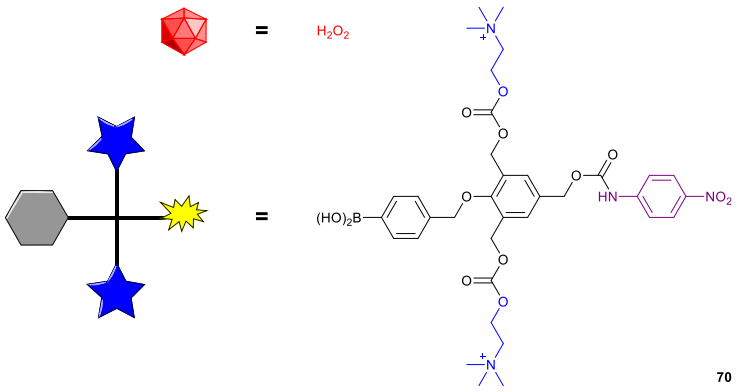
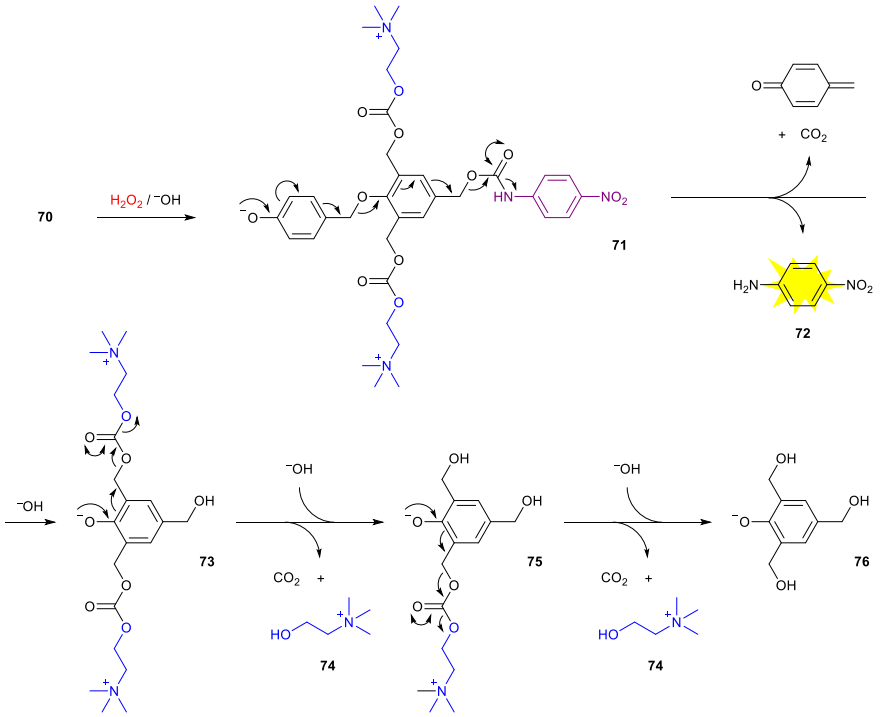


Figure 4 Structure of dendron 70; responsive to target, and signal propagating molecule, hydrogen peroxide.

The successful achievement of an autoinductive signal amplification methodology through the use of dendrimers was through the clever design of dendron **70**, which consists of a *p*-nitroaniline reporter molecule, two molecules of choline to relay the signal and a phenylboronic acid used for analyte recognition (Figure 4). In the presence of hydrogen peroxide, aryl boronic acids are known to undergo oxidation to their corresponding phenolates,[237] *via* a Baeyer–Villiger-type mechanism,[238] analogous to the Dakin oxidation.[239] This functional group transformation had previously been exploited for the fluorescent stoichiometric detection of hydrogen peroxide.[240]

In DCR, phenolate **71** produced as a result of the oxidation is unstable under the mild alkaline assay conditions and undergoes multiple quinone methide eliminations,[241] followed by entropically-favourable decarboxylation to release colourimetric reporter **72** and two equivalents of choline **74** to propagate the signal (Scheme 44). This is performed by the enzyme choline oxidase (COx),



Scheme 44 Breakdown mechanism of dendron 70 initiated by hydrogen peroxide.

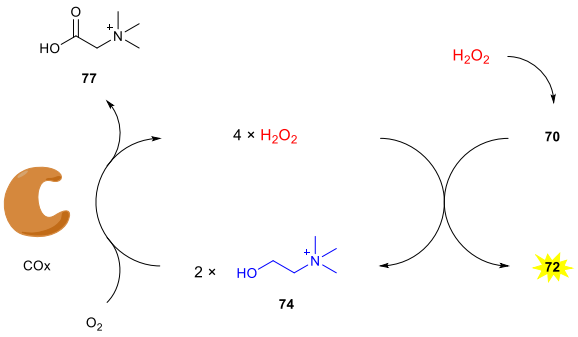
which twice oxidises choline to afford trimethylglycine **77** and in the process, generates two equivalents of hydrogen peroxide capable of initialising the breakdown of more molecules of dendrons **70** (Scheme 45). The requirement of COx for signal propagation is especially apparent at low concentrations of hydrogen peroxide, as a >50-fold signal enhancement is observed in comparison to a system without COx. The exponential production of hydrogen peroxide and subsequent exponential decay of all dendrons **70** leads to rapid signal production with full conversion observed after 50 minutes. The DCR was then applied to the colourimetric detection of penicillin-G-amidase (PGA), through the use of a choline-releasing probe, and showed a significant signal increase in comparison to a classic probe-based approach, allowing for PGA concentrations of 0.1 μgmL^{-1} to be detected.

One of the major benefits of using DCR as a signal amplification technique is its modularity. For example, by changing the boronic acid trigger with a silyl-protected phenol enables an autoinductive exponential signal amplification procedure for the sensitive detection of fluoride ions.[242] Additionally, the enzyme amplifier could be replaced with other enzymes capable of producing hydrogen peroxide, such as alcohol oxidase (AOx).[243] Despite its wide application, a major weakness of DCR, like many exponential signal amplification methodologies, is the high background rate observed in the absence of the trigger, which arises from undesired hydrolytic cleavage of the carbonate linkages.[244] Another difficulty lies in the synthesis of the dendrimers as a large number of challenging synthetic steps that are often required to successfully obtain them.

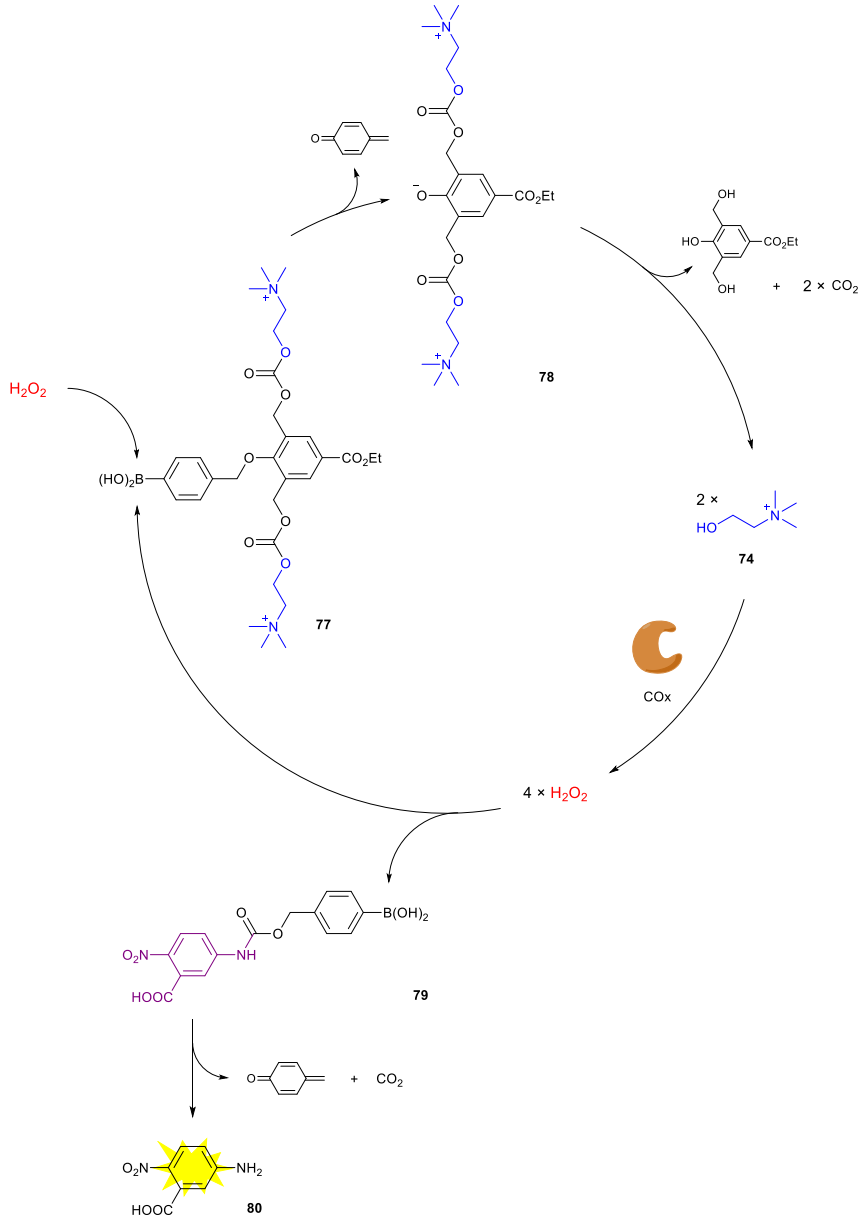
To address these issues, the group developed a two-component dendritic chain reaction (2CDCR) where one reagent **77** contains the trigger unit responsible for analyte detection and signal transduction while the second reagent **79** was solely responsible for signal production (Scheme 46).[245] Through this two-component methodology, the unwanted background signal was suppressed as hydrolysis of **77** does not directly lead to signal production. Also, by separating analyte detection and signal production into different compounds, a mix-and-match approach to sensing can be applied to allow DCR signal amplification to be applied to a fluorescence detection assay. Alternative enzyme amplifiers have also been incorporated into 2CDCR as a number of glucose-containing dendritic amplifier components were tested using glucose oxidase (GOx) as the enzyme amplifier.[246] Additionally, the use of a quinone-based detection component allowed for the 2CDCR to be applied to the detection of thiols.[247]

Autoinductive signal amplification can also be achieved in the absence of the enzyme amplifier by utilising the hydroquinone–quinone oxidation by molecular oxygen as a method for generating hydrogen peroxide *in situ* without the need for additional biological components.[248–249]

An example of a two-component autoinductive signal amplification protocol used in combination with target amplification for increased sensitivity was described by Baker and Phillips for the colourimetric detection of palladium (Scheme 47).[250] To achieve this autoinductive signal amplification methodology, the first component **81** of the two was equipped with an allyl carbamate trigger, for selective palladium detection, and two fluoride leaving groups as the amplified signal relay. In the presence of palladium therefore, catalytic deallylation and decarboxylation occurs to give 4-(difluoromethyl)aniline which undergoes a double 1,6-elimination to produce two fluoride ions. The second component **82**, a colourimetric probe for fluoride detection itself,[251] was equipped with a silyl protecting group, sensitive to the



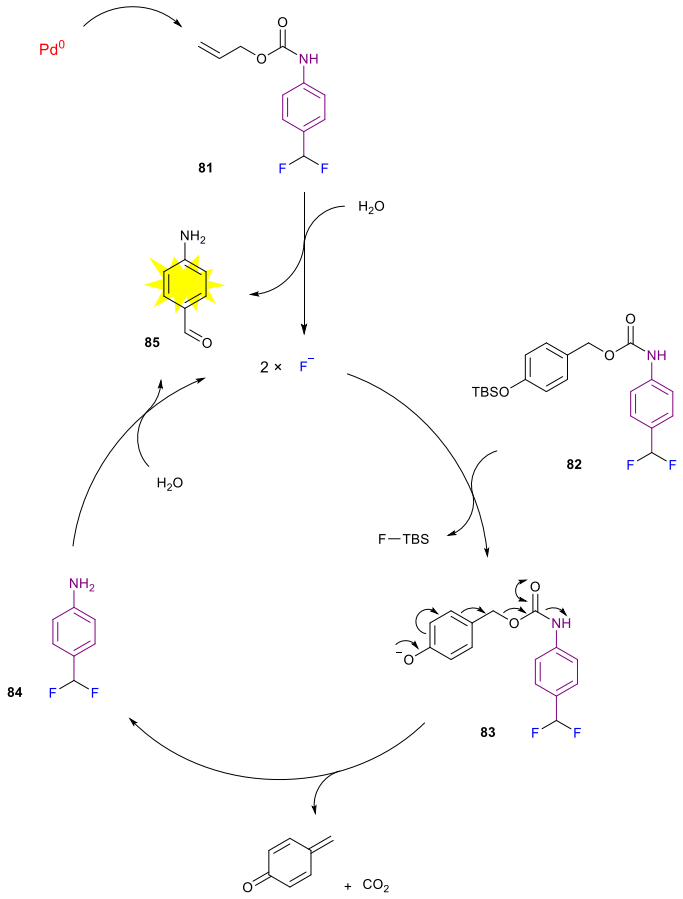
Scheme 45 Signal propagation in DCR: enzyme-catalysed hydrogen peroxide formation.



Scheme 46 Two-component dendritic chain reaction (2CDCR).

fluoride relay signal, along with another two equivalents of fluoride to further amplify the signal. After initial breakdown of **81** in response to the analyte, silyl deprotection of **82** occurs due to the generated fluoride and intermediate phenolate **83** also undergoes a 1,6-elimination and decarboxylation to afford another equivalent of aniline **84**, capable of eliminating further to afford another two equivalents of fluoride. Colourimetric detection occurs following elimination of **84** as the compound 4-aminobenzaldehyde **85** produced as the elimination by-product is yellow. This two-component autoinductive signal amplification strategy in combination with an analyte that exhibits target catalysis allows for a LOD of 0.36 ppm palladium, a concentration of palladium typically found in roadside dust.[252]

The reagents used to within this assay are thermally-stable and inexpensive and the colour produced can be seen with the naked eye enabling this methodology to be used in resource-limited environments. However, reaction times are slow with >24 hour assay times required to detect palladium concentrations close to the LOD. Also, due to the slow hydrolysis of **82**, an amplified background reaction limits assay sensitivity, although the introduction of allylic ethers as elimination linkers have shown promise in minimising background reactions yet maintaining signal propogation.[253] The modularity of the small molecule autoinductive signal amplification approach has enabled an extension of compound **82** to be developed for fluorescent fluoride detection.[254]



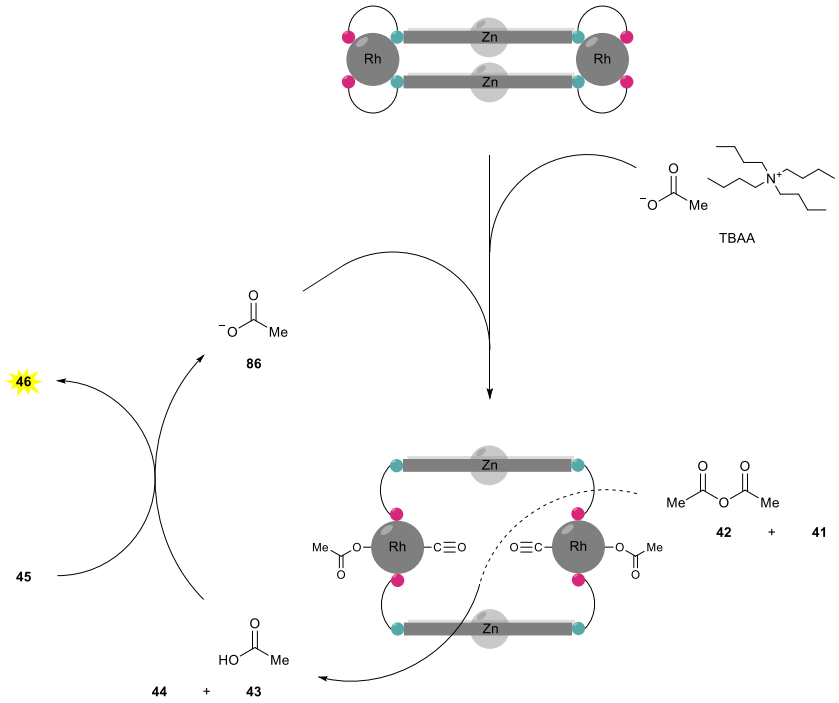
Scheme 47 Two-component small molecule autoinductive signal amplification for colourimetric palladium detection.

The ability of a signal amplification protocol to generate products that not only provides a detectable signal but can also increase the rate at which the signal is produced, has allowed previously inaccessible detection limits to be achieved. Inspired by DCR, the majority of autoinductive signal amplification approaches currently described revolve around small molecule-based systems which allow for thermally-stable, inexpensive reagents to be used for analyte detection, signal amplification and signal production in a simple manner. Current challenges involve improving reaction times to allow for these signal amplification methodologies to be applied within the POC setting. In addition, suppression of the high background rates associated with spontaneous hydrolysis of these reagents should allow for better detection limits to be obtained.

Autocatalytic Signal Amplification

Autocatalysis is the process whereby the product of a reaction is able to catalyse the production of itself.[255] This fascinating phenomenon has been intensively studied for the past forty years since autocatalysis may have played a significant role in the origins of life.[256–257] Autocatalytic mechanisms can vary considerably from templated self-replication and physical reproduction processes to asymmetric autocatalysis.[258] Early synthetic examples of autocatalysis involved the self-replication of small oligonucleotides where the product can act as a template, assisting the formation of more oligonucleotides.[259–261] Synthetic template-assisted autocatalysis,[262–263] has also been applied to the self-replication of peptides,[264] as well as small molecules.[265] Metal-catalysed autocatalysis was first described by Soai *et al.* where the alcohol formed as the product was able to act as a ligand within a zinc-catalysed alkyl addition to aldehydes, promoting the formation of more of itself.[266] Since the reaction can be performed asymmetrically, this reaction can be applied to selectively amplify one enantiomer over another and therefore increase the enantiometric excess of a near racemic mixture.[267–270] This powerful asymmetric amplification method has even been used to discriminate between carbon-12/carbon-13 chirality,[271] which has huge implications for determining the origins of homochirality.[272–273]

Although considerable effort has been made towards enantioselective amplification using autocatalysis,[274] the application of autocatalysis towards a signal amplification methodology has been comparatively less explored. To date, only two autocatalytic

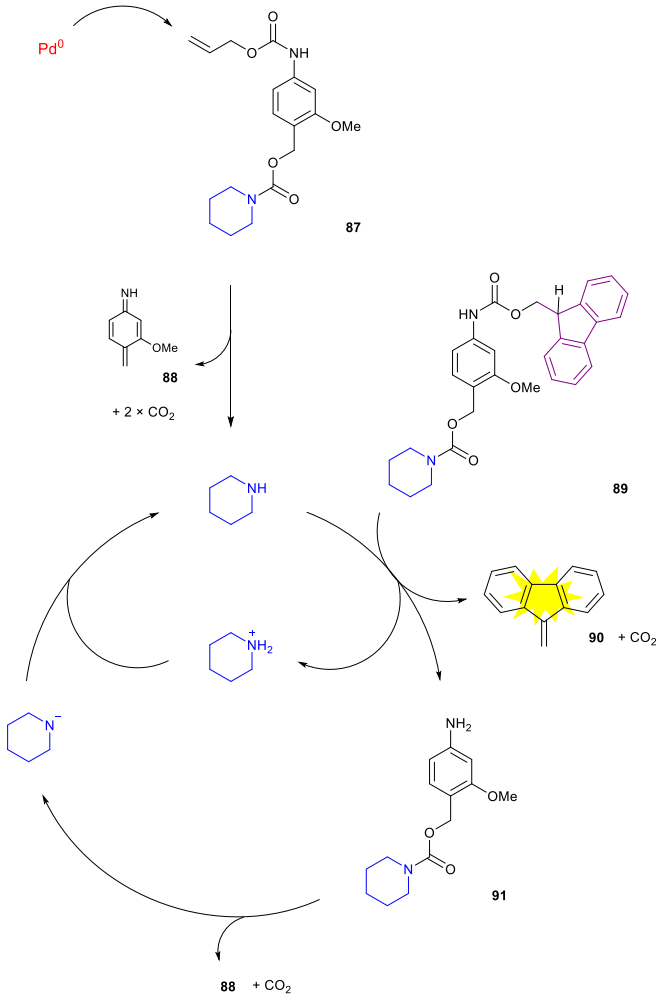


Scheme 48 Supramolecular autocatalytic signal amplification for fluorescent acetate detection.

signal amplification methodologies for sensing have been published and both are advancements upon signal amplification concepts already described herein. The first of the two was developed by Yoon and Mirkin who manipulated their supramolecular catalysts to be activated by the product allowing for autocatalytic signal amplification to be applied towards the fluorescent detection of acetate anions (Scheme 48).[275]

In a previous example, supramolecular constructs based upon the WLA were shown to rearrange selectively in the presence of the chloride analyte to reveal a bimetallic active site for a Lewis acid-catalysed acylation reaction. Since the by-product of this acylation is acetic acid, the authors hypothesised that an autocatalytic variant of SAAC could be achievable if the produced acetate anion could bind to the allosteric site of the pre-catalyst and also induce ligand rearrangement. Thus, in the presence of tetrabutylammonium acetate (TBAA), used as the model analyte, and under a carbon monoxide atmosphere, catalytic activity was shown to be switched on through the production of fluorescence. Initially, a minor amount of the catalysts are activated leading to slow product formation. However, as more acetate is produced, an increase in catalyst activation is observed leading to an acceleration of catalytic activity until the reaction reaches its saturation point when full consumption of the substrate occurs. This is confirmed by a study of the reaction kinetics; the conversion–time graphs display sigmoidal curves characteristic of autocatalytic product generation which starts with a slow induction period followed by an exponential increase before reaching a plateau.[276] Similarly to PCR, analyte concentration correlates with the time taken to reach exponential signal amplification. Importantly for autocatalytic signal amplification protocols, almost no conversion was observed in the absence of acetate. Although variations at the allosteric site of the catalyst amplifier and the use of alternative pH-responsive reporters for different readouts would allow this autocatalytic signal amplification protocol to be used within multiple applications, the current procedure described appears to be limited towards only acetate detection.

A more flexible approach to autocatalytic signal amplification was provided by Phillips *et al.*, who described an autocatalytic variant of their two-component autoinductive signal amplification procedure for colourimetric palladium detection (Scheme 49).[277] To achieve autocatalysis, signal amplification reagent **89** was designed, inspired by amine proliferation compounds used within photolithography,[278] with a 9-fluorenylmethoxycarbonyl (Fmoc) reporter moiety and a piperidine unit connected *via* an aniline linker. Analyte-responsive reagent **87** was equipped with an allyl carbamate trigger for selective palladium detection adjoined with a signal propagating piperidine group *via* an electron-rich aniline, which facilitates elimination upon analyte recognition.[279] In the presence of palladium, deallylation and elimination of **89** occurs releasing the piperidine catalyst. Since palladium also exhibits target catalysis, multiple equivalents of piperidine can be released *per* molecule of palladium thereby increasing assay sensitivity. After release, piperidine acts as a catalytic base, deprotonating **89** to release another equivalent of piperidine and the dibenzofulvene chromophore **90**. Proton transfer then occurs regenerating the original catalytic base and creating another catalyst. This cycle repeats until full consumption of signal amplification reagent **89** occurs and full conversion is obtained.



Scheme 49 Small molecule autocatalytic signal amplification for colourimetric palladium detection.

A piperidine catalytic loading of 0.1 mol% was shown to initiate full breakdown of **89** over 18 hours and exhibited a sigmoidal reaction profile over this time period which indicates that an autocatalytic mechanism was in effect. This two-component small molecule approach to autocatalytic signal amplification was capable of reaching a LOD of 12 ppm for palladium and variations of 2 ppm in palladium concentration could be easily distinguished. Despite >1000-fold signal amplification, the long induction time required for low analyte concentrations prevents this methodology from being employed for POC diagnostics. However, this methodology could see use within areas such as stimuli-responsive materials where high amplification is required but a specific timeframe in which this occurs is less important.[280]

Autocatalytic signal amplification has the potential to fulfil the needs of diagnostic assays which suffer from a lack of sensitivity and is capable of detecting analyte concentrations unreachable by other signal amplification methods. The development of such protocols is still in its infancy with only two examples successfully demonstrated to date. They have however, shown that autocatalytic signal amplification is achievable through completely different methods and should inspire the next influx of amplification approaches to match, if not better, current detection limits. Future efforts should also be aimed at providing autocatalytic signal amplification towards detection of biological samples allowing them to be incorporated within medical diagnostic applications. Furthermore, the time taken to induce exponential amplification should be dramatically shortened to enable autocatalytic signal amplification to be applied at the POC setting.

Receptor Amplification

Within a detection assay, signal production typically occurs after the result of an analyte–probe recognition event. Increasing the number of analyte receptors upon the probe would therefore lead to an increase in the probability of an analyte–probe recognition event occurring and therefore faster signal production. This concept is perfectly exemplified by Amir and Shabat who were able to

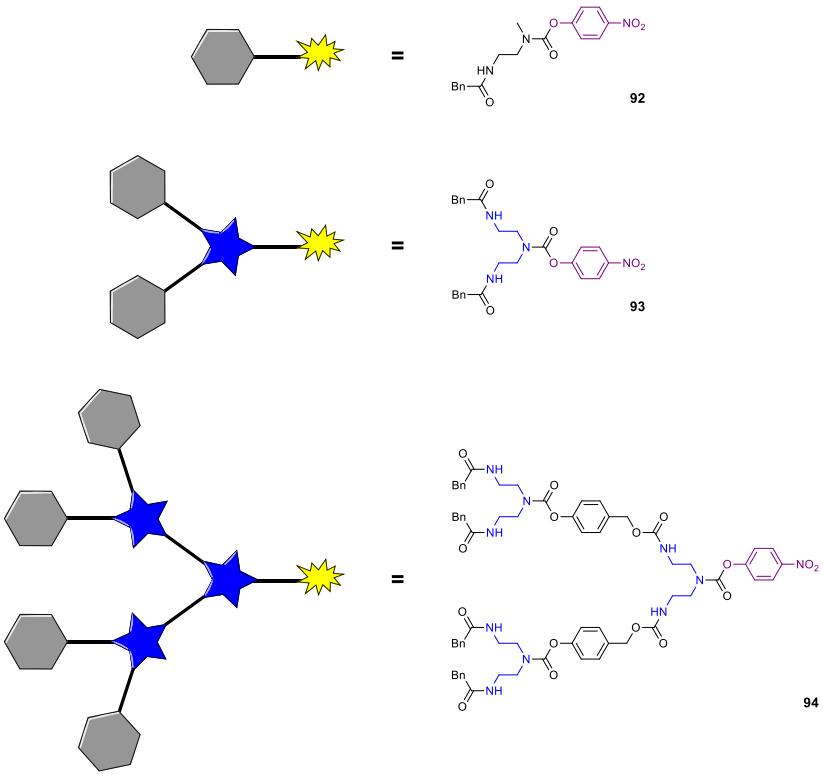


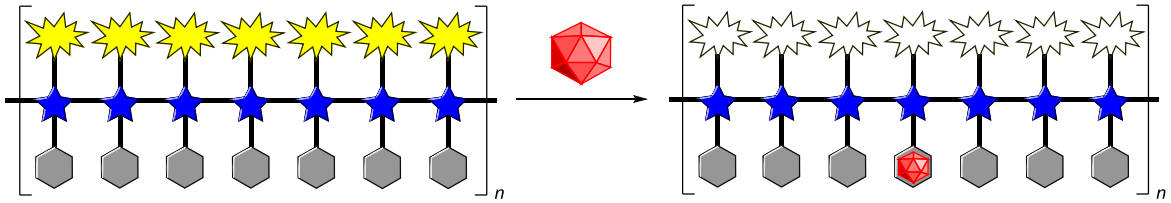
Figure 5 Concept of receiver amplification and the structures of dendrimers 92, 93 and 94.

increase the number of analyte-responsive triggers on a dendrimer in order to achieve an amplified signal response (Figure 5).[281] In this example, three dendrimer derivatives were synthesised containing one, two or four enzyme responsive triggers connected to a single reporter molecule. Since the removal of just a single trigger is capable of releasing the reporter molecule, the dendrimer with the most receptors should deliver the fastest response. Indeed, in the presence of the enzyme target, dendrimer **93** with two enzyme triggers was shown to eliminate twice as fast as dendrimer **92** containing only one enzyme trigger. However, due to slow elimination kinetics associated with the increased number of eliminations required, dendrimer **94** with four enzyme triggers attached to the reporter molecule was actually shown to produce a considerably slower signal. Still, this dendritic approach to receptor amplification was successfully coupled to a dendritic signal amplifier to significantly reduce the time-to-result yet still provide an amplified signal for the detection of PGA.[282] It is on this basic principle that the majority of receptor amplification methodologies are based upon.

Similarly, but conceptually opposite, to label amplification, receptor amplification methodologies utilise the multivalent characteristics of a range of molecular structures to sufficiently amplify the number of receptors adjoined to the signal producer. This has allowed structures such as polymers, micelles and hydrogels to all be successfully employed as receptor amplifiers within sensing methodologies.

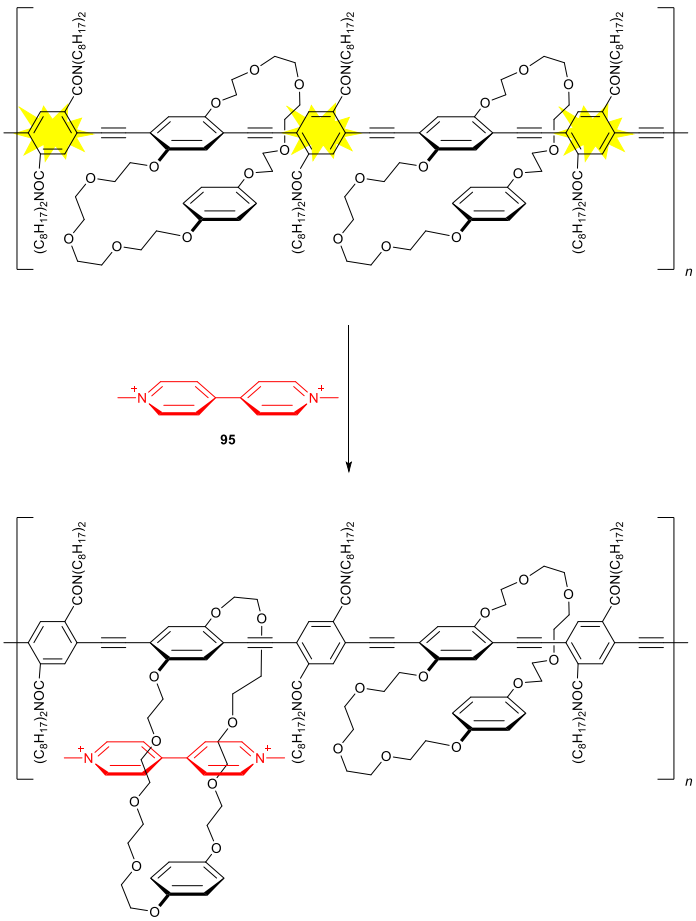
Polymers

Prior to the introduction of self-immolative dendrimers, the development of conjugated polymers was fundamental to enabling receiver amplification as this allows communication between repeating units of the polymer.[283] Functionalising the conjugated polymers, known as “molecular wires”, with analyte-responsive receptors generates a single compound with multiple linked receptors. However, it was the use of fluorescent conjugated polymers developed by Swager *et al.* that adapted the molecular wire approach to be used for receptor amplification within a sensing application (Scheme 50).[284]



Scheme 50 The molecular wire approach to receptor amplification.

In a seminal publication, Zhou and Swager described the use of cyclophane receptors attached to a polyphenylacetylene backbone for the fluorescent detection of paraquat (PQ) (Scheme 51).[285] A strongly fluorescent molecular wire was synthesised through palladium-catalysed cross coupling of 4-iodophenylacetylene monomers equipped with bis(*p*-phenylene)-34-crown-10 (BPP), an excellent binder of PQ.[286] In the presence of the analyte **95**, PQ was shown to dramatically quench the fluorescence of the conjugated polymer in comparison to the monomer. In fact, a 65-fold increase in quenching was observed through the

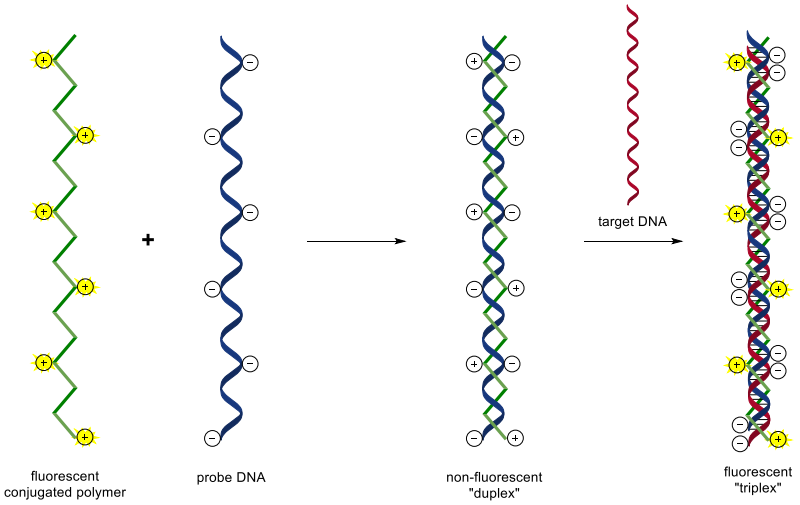


Scheme 51 The molecular wire approach for the fluorescence detection of PQ.

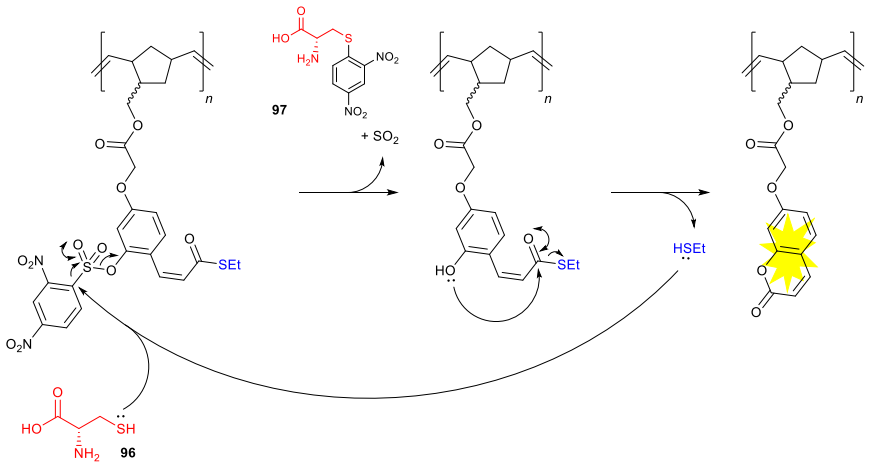
molecular wire approach, which was attributed to energy migration transmitted throughout the polymer. In other words, a single analyte binding to a single receptor is able to exhort fluorescence quenching over the entire polymer. At high molecular weight polymers though, a marked decrease in sensitivity is observed due to the length of the polymer being longer than the diffusion length of excitation. Attempts to increase the diffusion length of excitation by enhancing the delocalisation throughout the polymer backbone in order to improve sensitivity were unsuccessful.[287] Increased sensitivity can be achieved however through deposition of the molecular wires into thin films as energy migration can then occur between individual polymers.[288]

The concept of receiver amplification employed by this method has allowed amplification to be applied to the large array of simple analyte receptor systems previously developed, enabling lower LODs to be obtained through these conjugated polymer-based chemical sensors.[289] The considerable number of polymerisation techniques, as well as the number of analyte receptors available, has meant that the amplified detection of small ions, explosives and biomolecules can be achieved using fluorescent conjugated polymers.[290] Not only this, but modification of the polymer backbone has allowed for colorimetric derivatives of receiver-amplified polymer-based detection methods to become possible allowing analyte detection to be achieved with the unaided eye.[291]

Typically, conjugated polymers display fluorescence and it is in the presence of the analyte that fluorescence is quenched. In contrast, Leclerc *et al.* demonstrated the use of conjugated-polymers for signal-amplified switch-on fluorescent nucleic acid detection (Scheme 52).[292] In this invention, a cationic, water soluble, thiophene-derived conjugated polymer,[293] and an anionic, complementary strand of probe DNA initially come together through electrostatic interactions. The complexes then aggregate together because of their poor solubility in aqueous media and due to the close proximity of the polymers, as well as polymer stiffening, fluorescence quenching is observed.[294] In the presence of target DNA, hybridisation preferentially occurs with the probe DNA and a stable triplex is formed where the conjugated polymer is wrapped around the DNA double helix, bound to the anionic phosphate backbone. The resulting uniform, helical structure of the conjugated polymer, along with the triplex being more soluble in water, leads to dequenching and a switch on of fluorescence. This technique was shown to be surprisingly selective as well as sensitive since a two base pair mismatch can be easily discriminated and a LOD of 2.4 aM after an assay time of 1 hour can be achieved. Labelling the probe DNA with a fluorophore whose optimal absorption wavelength is identical to the emission wavelength of the conjugated polymer can provide FRET enhancement within the detection methodology.[295] This not only improves selectively since a single base pair mismatch could be distinguished, but also improves sensitivity as a LOD corresponding to 3 zM could be achieved after just



Scheme 52 Conjugated polymers for fluorescence detection of nucleic acids.



Scheme 53 Stimuli-responsive polymeric films for fluorescence thiol detection.

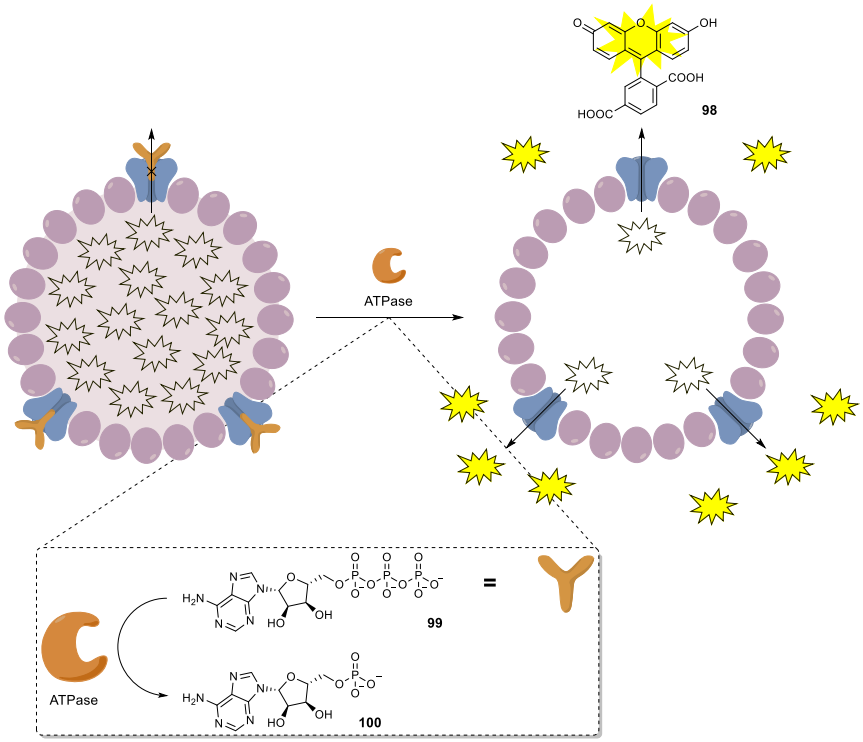
a 5 minute assay time. This increase in sensitivity is attributed to additional fluorescence signal amplification (FSA),[296] as one conjugated polymer is capable of exciting a large number of fluorophores.[297]

The ability to transform stimuli-responsive polymers into smart surfaces capable of delivering inherent analyte detection and response amplification simultaneously has been an attractive research goal with a number of potential applications.[298] As such, Phillips *et al.* have recently been in the process of developing stimuli-responsive polymeric materials capable of converting localised signals into global macroscopic responses.[299] In order to achieve an application within sensing, these polymeric materials were then coupled with a signal producing mechanism enabling a local molecular detection event to be translated into a macroscopic fluorescent response (Scheme 53).[300] To achieve this, a non-fluorescent polymeric film was first synthesised armed with analyte-responsive triggers specifically designed for thiol detection. In the presence of the target analyte, in this case L-cysteine **96**, selective deprotection of the nosyl groups occurs revealing a phenol capable of undergoing an intramolecular cyclisation reaction to generate a fluorescent coumarin moiety.[301] In the same instance, an equivalent of ethanethiol is also released and is able to react with neighbouring nosyl protecting groups upon the polymer, thus propagating the signal and amplifying the response. A non-fluorescent film covering 500 mm^2 was transformed to complete fluorescence in response to a 0.1 mM concentration of cysteine after 48 hours. Despite the long assay time required to achieve full conversion at low analyte concentrations, minimal background reactions in the absence of the target were also observed demonstrating sufficient material stability.

Encapsulated Reagents

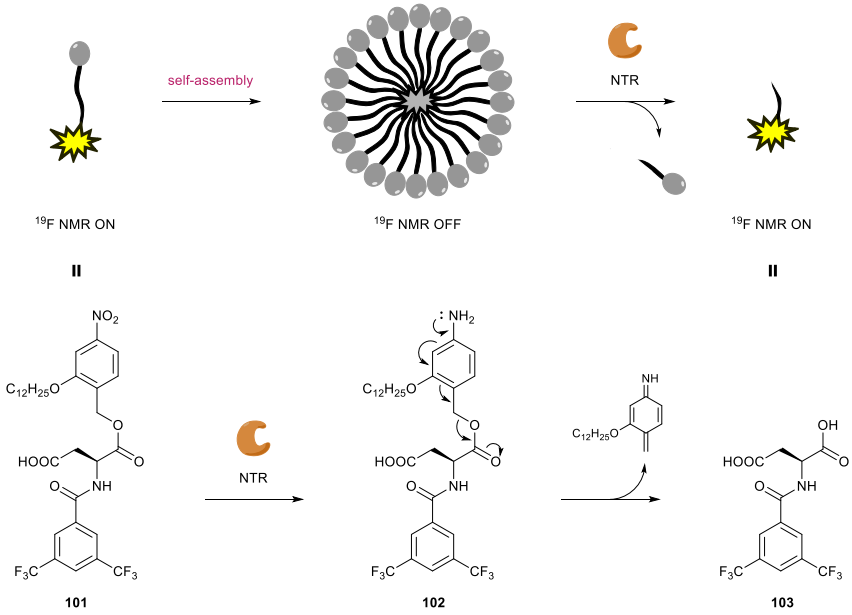
The concept of encapsulation to segregate reaction reagents and slowly release them in response to product formation for reaction rate acceleration is a well-known method of providing chemical amplification.[302–303] Manipulating this premise towards a sensing methodology requires modification of the capsule to release its contents selectively in the presence of the analyte and a change in physical property must accompany content release in order for effective analyte detection.

The discovery of fluorophores that undergo self-quenching at high concentrations,[304] such as fluorescein,[305] has enabled the development of 'switch-on' fluorescent sensing methodologies utilising polymeric assemblies and supramolecular structures that display stimuli-responsive fluorescence emission characteristics.[306] In a prime example of this, Matile *et al.* described the use of synthetic supramolecular pores within vesicles for the fluorescent detection of enzyme activity (Scheme 54).[307] Artificial rigid-rod molecules capable of self-assembling into β-barrels,[308] were



Scheme 54 Fluorometric detection of enzyme activity with synthetic supramolecular pores.

constructed and then filled with anionic enzyme substrate adenosine triphosphate (ATP) **99**, which can bind to the cationic inner wall of the barrel through electrostatic interactions. The blocked barrels were then slipped into the lipid bilayer of a vesicle containing a high concentration of 5-carboxyfluorescein **98** and as such, were non-fluorescent. Since a large number of synthetic pores, or analyte receptors, are present *per* vesicle and only one is needed to be unblocked to release its cargo then significant receptor amplification is demonstrated by this approach. In the presence of the analyte, a nonspecific adenosine triphosphatase (ATPase) enzyme,[309] dephosphorylation of ATP **99** to adenosine monophatase (AMP) **100** occurs. Due to the decrease in size and the reduced number of anionic binding sites, AMP is unable to sufficiently block the barrel and is expelled. Once the barrel is unblocked, this allows the release of the trapped molecules within the vesicle through the synthetic pores and into the reaction medium. Consequently, dilution of 5-carboxyfluorescein **98** prevents self-quenching leading to the observed turn-on of fluorescence.

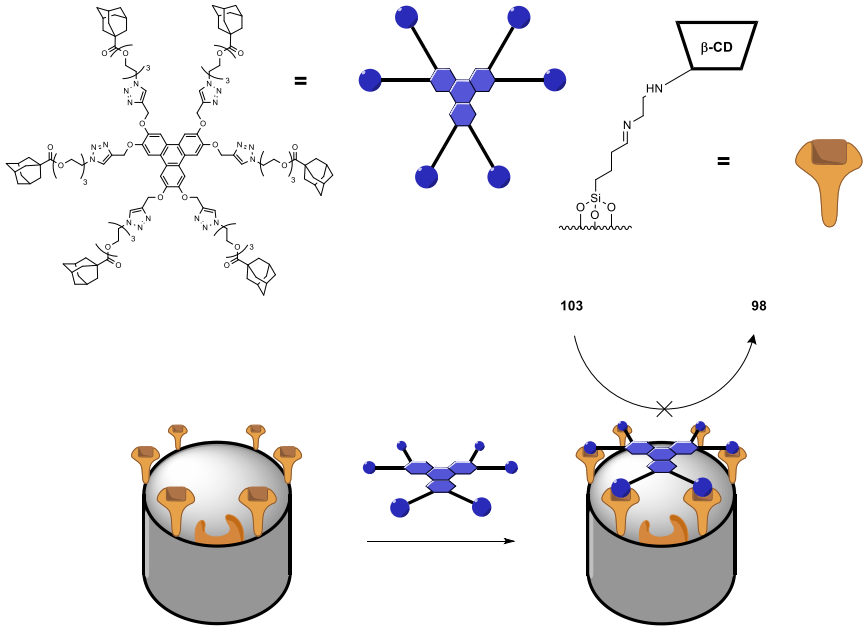


Scheme 55 Receptor amplification through self-assembling probes for the 19F NMR detection of enzyme activity.

The versatility of this amplification methodology was also shown as it was easily adapted to the detection of other enzymes, such as aldolases and phosphatases, through the use of the appropriate unmodified enzyme substrates as pore blockers. However, the advantage of not requiring labelled enzyme substrates is highly dependent on the enzyme substrate showing high affinity for the pores and the enzyme product having a significantly lower affinity. In particular, to enable vesicle receptor amplification for the fluorescent detection of ATP, an analyte detection method without synthetic pores had to be used.[310] Despite this, the rigid-rod approach for the development of synthetic, multifunctional pores capable of responding to changes in pH, concentration and voltage,[311] has enabled a variety of different analytes to be detected using synthetic and bioengineered membranes.[312]

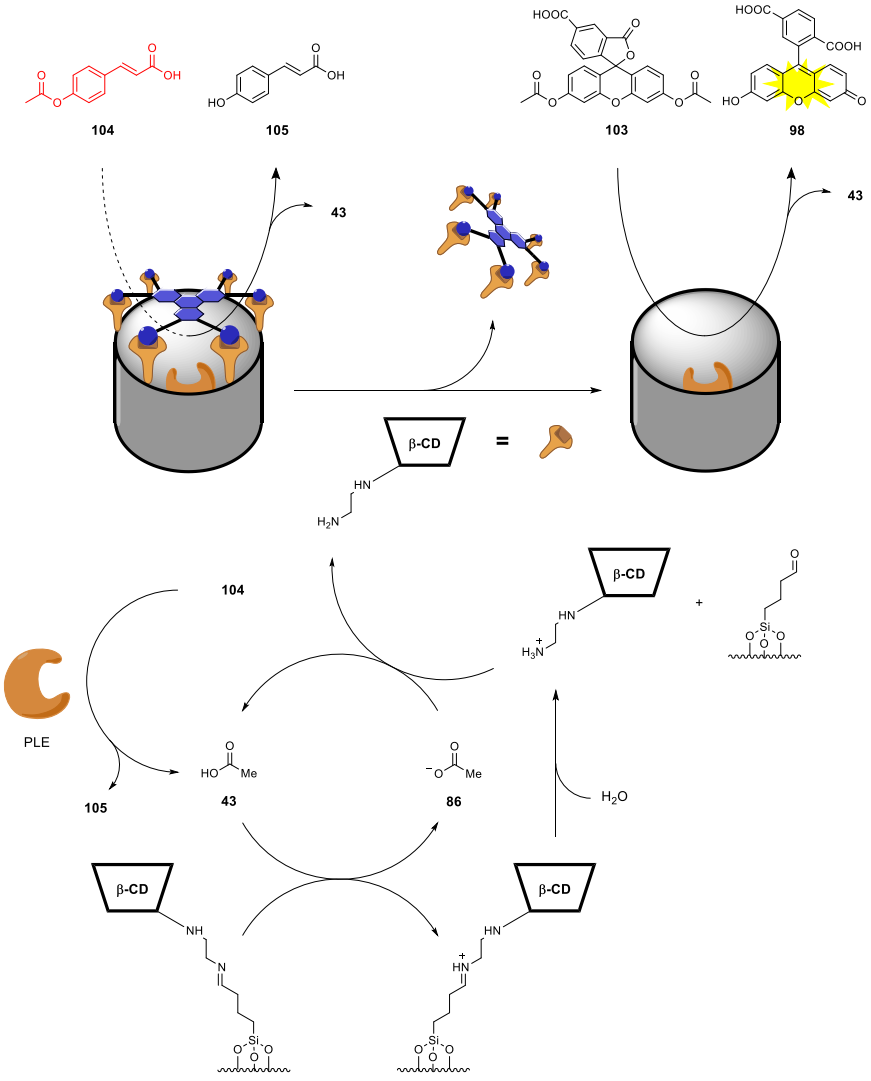
Similarly to self-quenching fluorophores, the phenomenon of molecular signal changes at high label concentrations also occurs with self-assembling fluorine-containing compounds when using ^{19}F nuclear magnetic resonance (NMR) as the detection method. Developed by Hamachi *et al.*, a detailed study was conducted into the different combinations of monomers that could be obtained through modification of the fluorine-containing tail group, the linker and the analyte-responsive head group and the effect this would have upon self-assembly.[313] The successful formation of spherical, self-assembled nanoprobes, with the fluorine-containing tail-groups on the inside, was determined by a switch-off of the ^{19}F NMR signal. Since the outside of the nanoprobe is covered with the analyte-responsive head groups and modification or removal of just one could disrupt aggregate formation, then considerable receptor amplification can be achieved.

To adapt this observation towards a sensing methodology, the group used nitrobenzene head groups for the ^{19}F NMR detection of the enzyme nitroreductase



Scheme 56 Structure of both the size-selective gate and the hinge, and their use in the construction of pH-controlled, enzyme-encapsulated, mesoporous silica nanoparticles.

(NTR) (Scheme 55).[314] In this approach, enzyme-catalysed reduction of the nitro group upon probe **101** to the corresponding aniline **102** generates an unstable intermediate that undergoes 1,6-elimination to afford reporter molecule **103**. Subsequently, disaggregation of the nanoprobes occurs and a switch-on of the ^{19}F NMR signal is observed. Importantly, due to the designed elimination procedure, a shift in the ^{19}F NMR signal is also observed which confirms the detection of enzyme activity *via* the proposed mechanism as opposed to nanoprobe disaggregation occurring through other means. The adaptability of this technique was also demonstrated as by changing the head group to a specific peptide chain, the amplified ^{19}F NMR detection of cancer biomarker MMP2 could be achieved. The nanoprobes also exhibit excellent biocompatibility and this method has been demonstrated for both cell surface,[315] and intracellular protein imaging.[316]



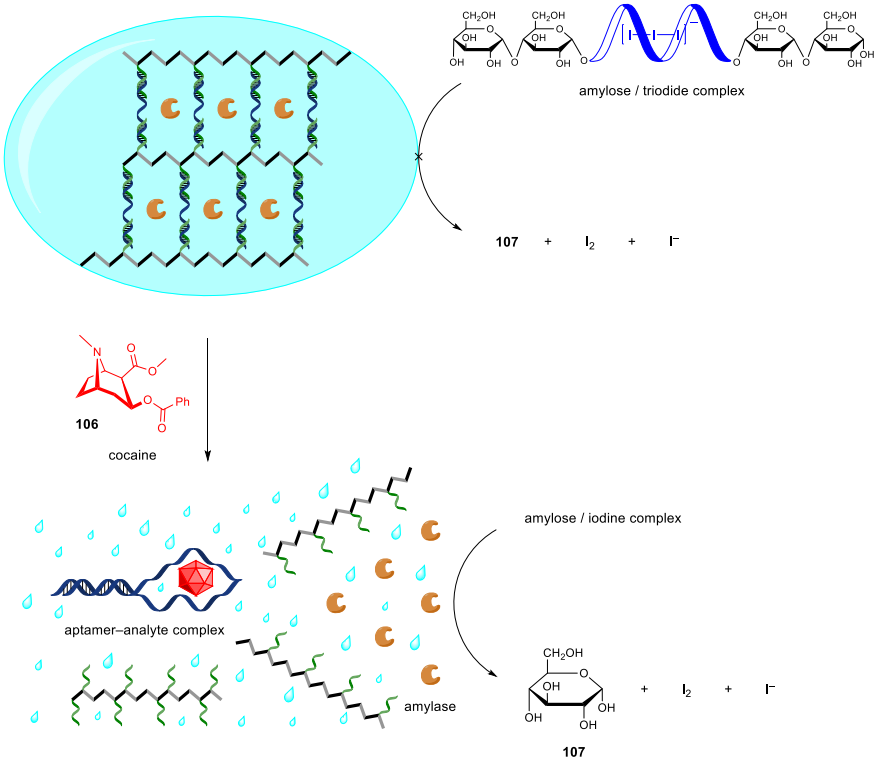
Scheme 57 Size-selective, pH-controlled, enzyme-encapsulated mesoporous silica nanoparticles for the fluorescence detection of small, acylated compounds.

Stimuli-responsive release of encapsulated reagents,[317] has enormous application to a number of biomedical applications such as drug delivery and self-healing materials.[318] As such, a considerable number of analyte-triggered reagent release methodologies are not necessarily designed for sensing purposes.[319–320] Recently, advances have been made from encapsulated reagents to encapsulated enzymes in an effort to provide further amplification as well as to achieve programmed enzymatic reactions.[321] From a sensing perspective, one particular contribution of note was provided by Xue and Zink who demonstrated an enzyme chemical amplifier using mechanised nanoparticles.[322]

In this method, size-selective pH-controlled silica nanoparticles were constructed containing porcine liver esterase (PLE) and capped with a star-like gate through imine hinges (Scheme 56).[323] The gate allows the coexistence of the enzyme and enzyme substrate in the same media yet prevents their interaction through size-exclusion. System activation occurs in the presence of the analyte, 4-acetoxycinnamaldehyde (ACA) **104**, which is small enough to fit through the gate and is subsequently hydrolysed by the enzyme (Scheme 57).[324] The acid produced as a result of enzyme hydrolysis is then capable of catalysing the hydrolysis of the imine hinges holding the gate in place. Once all the linkages are broken, the gate is removed from the nanoparticle which therefore allows interaction between the enzyme and pro-fluorescent enzyme substrate, 5-carboxyfluorescein diacetate (CFDA) **103**. Enzyme-catalysed hydrolysis of enzyme substrate CFDA not only allows enzyme activity to be measured by fluorescence but also produces further equivalents of acid, which leads to further gate removal and therefore an increased rate of fluorescence production. Although the system demonstrated autoinductive amplification, reaction times were long owing to the slow turnover rate of the enzyme. Also, this methodology is limited to the detection of acylated compounds that are small enough to fit through the gaps in the gate. Despite this, background rates are negligible showing that enzyme activity can be effectively controlled using size-selectivity and this methodology could have wider application within biosensing due to both the high biofunctionality and biocompatibility of mesoporous silica nanoparticles.[325]

Hydrogels

Hydrogels are hydrophilic polymer networks that can absorb up to 99% of their own weight in water without dissolution.[326] They possess the ability to contain a large number of analyte receptors either physically encased within the polymer matrix or covalently bound to the polymer and can induce a considerable physical response in the presence of specific stimuli.[327] This, along with their high biocompatibility,[328] has seen the inclusion of hydrogels within a number of sensing applications such as temperature and pH monitoring,[329] as well as for the detection of biologically-related material.[330] Typically, the physical response elicited is either through swelling or shrinkage of the hydrogel and this response can be measured through changes in optical transmission,[331] or refractive index.[332] However, these methods require sophisticated instrumentation to afford an accurate readout and as a result, there has been efforts made to induce complete disintegration of the hydrogel in response to an analyte allowing for naked eye detection. Furthermore, by accompanying full dissolution of the hydrogel with a colour change, then an unambiguous positive result can be easily determined by eye. One such methodology was developed by Tan *et al.*, who developed an aptamer-based colourimetric hydrogel platform for the visual detection of cocaine **88** (Scheme 58).[333]

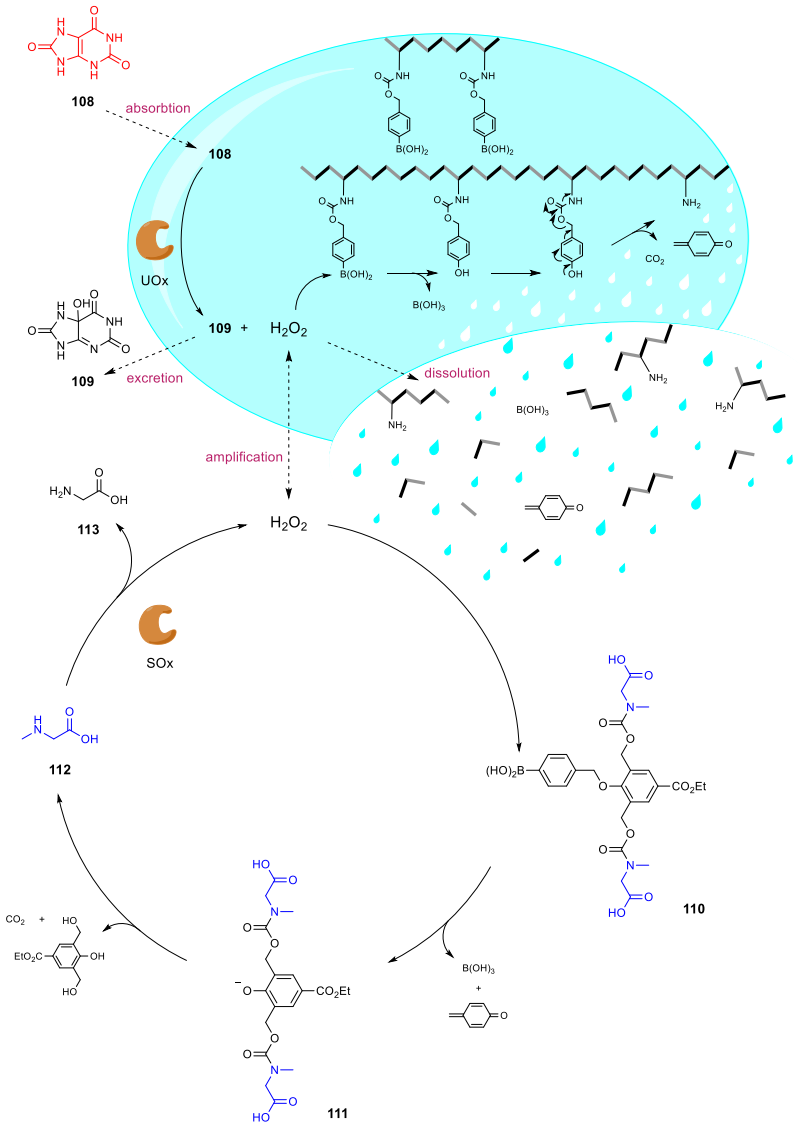


Scheme 58 An aptamer-bound, enzyme-encapsulated hydrogel for colourimetric cocaine detection.

In this procedure, amylase enzyme amplifiers were trapped within a cross-linked polymer hydrogel that was dependent on DNA aptamers, that are selective for cocaine **106**, for structural support.[334] In the presence of the analyte, competitive binding for the aptamers occur leading to hydrogel destabilisation and then inevitable dissolution of the hydrogel with concomitant release of amylase enzymes. Thus, the enzyme-catalysed breakdown of amylose is initiated and when the assay medium is stained with iodine, the characteristic blue colour associated by the presence of amylose does not appear.[335] The combined receiver amplification of the hydrogel and the signal amplification delivered by the enzyme enabled cocaine detection to less than 20 ng with the naked eye within 10 minutes. Due to the use of DNA aptamers for analyte detection high selectivity for cocaine over structurally similar compounds such as benzoylecgonine (BE) and ecgonine methyl ester (EME) was also observed. Enzyme trapping, compared with some enzyme conjugation methods, is extremely mild yet effective as no loss of enzyme activity was seen between trapping and activation. Also, no undesired substrate breakdown was seen in the absence of the analyte since the hydrogel and the enzyme substrate are both polymers. As glucose **107** is produced as a result of the enzyme-catalysed breakdown of amylose, cocaine detection can also be measured quantitatively with a commercially available personal glucose meter.[336]

Supramolecular hydrogels, hydrogels constructed solely on the self-assembly of small molecule hydrogelators rather than through chemical cross-linking or polymerisation, are becoming increasingly popular because in addition to being able to support biomaterial within the hydrophilic gel, hydrophobic molecules such as fluorophores can also be supported within the supramolecular framework itself.[337] As such, a considerable number of supramolecular hydrogels have been used for a number of fluorescent chemosensors that respond to molecular recognition and enzyme activity.[338] Of particular note are the supramolecular hydrogel-based sensing techniques developed by Hamachi *et al.* that have been shown to be easily adaptable towards the fluorescent detection of polyanions,[339] polycations,[340] and PSA.[341] However, continuing the endeavour for analyte detection without instrumentation, the group very recently successfully coupled their chemically reactive supramolecular hydrogel with a DCR-type amplification procedure for the naked-eye detection of uric acid in human plasma (Scheme 59).[342]

In this protocol, supramolecular hydrogels designed to be selective towards hydrogen peroxide, were constructed containing three components; analyte selective enzyme urate oxidase (UOx), signal transducing self-immolative compound **110**, and signal amplifying enzyme sarcosine oxidase (SOx). Despite the high number of encapsulated reagents, the hydrogels were shown to be



Scheme 59 DCR-enhanced, chemically-reactive supramolecular hydrogel for the visual detection of uric acid.

physically stable. In the presence of uric acid **108**, a biomarker for gout,[343] UOx catalyses its oxidation to 5-hydroxyisourate **109** producing hydrogen peroxide as a byproduct, which can go on to oxidise signal transducing reagent **110** to its corresponding phenolate **111**. This intermediate is unstable under the assay conditions and eliminates to produce two equivalents of sarcosine **112**. Signal amplifying enzyme SOx then catalyses the oxidative demethylation of sarcosine **112** to glycine **113** producing more molecules of hydrogen peroxide in the process. This can go on to react with more equivalents of **110** or react with phenylboronic acid residues upon the hydrogel framework. Oxidation and subsequent elimination of these residues is enough to destabilise the matrix and cause collapse of the hydrogel, a physical effect visible by eye. This system was capable of detecting 80 μgmL^{-1}, the threshold level of hyperuricemia, with the naked eye following a 3 hour incubation. The sensitivity was attributed to the amplification provided by DCR, allowing for a 5-fold decrease in analyte concentration that could be detected *via* this method. In addition, by swapping SOx with GOx, the detection method was easily adapted towards the detection of glucose. This mix-and-match approach using different enzymes allows the system to provide logic-gate output responses from different biological substance input signals.[344]

Conversely, signal production can also be attributed to hydrogel formation, rather than hydrogel dissolution, as Liang *et al.* recently demonstrated.[345] In this method, a tetrapeptide substrate for ALP was synthesised comprising of a phosphorylated tyrosine residue for target recognition and a fluorescein moiety to enable orthogonal enzyme detection to be achieved *via* fluorescence. In the absence of the enzyme, a transparent yellow solution is apparent and exhibits strong green fluorescence emission under UV irradiation. Dephosphorylation of the substrate was shown to occur selectively only in the presence of ALP and the hydrogelator product, once obtained in high conversion, self-assembles into nanofibres to form a hydrogel. In addition to the physical change of the assay, aggregation-induced quenching (AIQ) is also observed leading to a decrease in fluorescence. The assay exhibited a good dynamic range, obtained a LOD of 0.06 UmL^{-1} and could be used to quantitatively measure ALP activity within living cells.

Conclusions

Molecular amplification for sensing applications can now be achieved through a considerable variety of different approaches; whether focussed on target, label, signal or receptor amplification. The selection of which amplification strategy to choose is not necessarily a case of which one provides the greatest amplification but more a matter of which one is most suitable as part of a particular detection assay. This is dependent on the analyte being detected, the medium the analyte is being detected from, the medium the amplification is taking place in and the desired readout signal. Factors such as the required selectivity and sensitivity, as well as time and cost, also play an important role in selecting the most suitable amplification method.

Analyte detection methods are constantly evolving. Reversible affinity-based analyte recognition has made way to irreversible reaction-based analyte recognition in order to improve sensitivity. In addition; simple indicator-based approaches to sensing are

now being made obsolete by easy-to-perform, amplified detection assays. Recently, single-amplifier techniques have been elaborated with multiple amplifiers in a bid to achieve even greater sensitivity. Current efforts are being made not only towards the inclusion of multiple amplifiers but also the incorporation of multiple amplification approaches, such as target and signal amplification within the same detection assay. However, the strive to obtain lower and lower detection limits by more and more powerful amplification methods can lead to over-complication and problems with false-positive results from accidental activation. This is particularly true with regards to the attractive exponential amplification displayed by autocatalytic methods. Therefore, the ability to maintain low background signals as well as analyte selectivity is becoming increasingly important and not just sensitivity.

Future amplification methodologies will always aim for a detection limit of a single molecule within a complex mixture with the naked eye. This is not the only goal within sensing however as complex diseases, such as cancer, require the successful detection of a panel of biomarkers prior to diagnosis, not just one. Therefore, the ability for amplification methodologies to demonstrate multiplexed detection without interference is highly desirable. General reagent-based amplification strategies are also in high demand as the ability to choose an appropriate reagent for a specific analyte with a particular readout where amplification is guaranteed will prove to be very adventitious within clinical diagnosis. Previously, it has been highlighted that the best results are obtained in systems that rely on cascades of amplification events and that most amplification protocols rely on hybrid systems. Current objectives within sensing should therefore be to develop amplification cascades that utilise both biological and synthetic components to utilise selectivity, sensitivity and versatility within analyte detection assays.

Acknowledgements

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Notes and references

§ Despite containing similarities with target catalysis, this particular sensing methodology has been designed for the detection of copper ions. Therefore, palladium is not the target analyte and its catalytic properties are employed for signal amplification instead. As such, this is an example of single-catalyst signal amplification as opposed to target amplification as the target metal ions in this case themselves exhibit no catalytic properties and are therefore not considered amplifiers.

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